IN VITRO TOXICITY OF ALUMINUM NANOPARTICLES IN RAT ALVEOLAR MACROPHAGES

THESIS

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AFIT/GES/ENV/06M-06

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Abstract

Nanomaterials, which are by definition in the 1 - 100 nanometer range, have numerous possible benefits to society, but currently there is a lack of data that characterizes these materials effects on human health and environment. In general nanomaterials are of interest to the Air Force because of their applications in electronics, sensors, munitions and energetic/reactive systems. Nanoparticles such as aluminum have been considered for enhancing propulsion in solid rocket fuel. To date, only a few studies have looked at the toxicological effects of direct exposure to nanoparticles, none with aluminum. It is important to increase the understanding of the nanomaterial exposure health impact before these materials are throughout diverse levels of occupations or fully used in large capacities within industry and the military. The purpose of this research is to observe and characterize the *in vitro* cellular effects of rat lung macrophages to exposure to aluminum oxide nanoparticles (Al_2O_3-NP) (30 and 40nm) compared to aluminum nanoparticles (Al-NP) (50, 80, and 120nm). This study concentrates on cell viability, mitochondrial function, phagocytosis ability, and cytokine response. Results indicate no to minimal toxicological effects on macrophages exposed as high as 500 μg/ml for 24 hours with Al_2O_3-NP. However, there was a significant delayed toxicity that occurred at 96 and 144 h post exposure. Al-NP indicate sight to moderate toxicity after 24 h exposure at 100 and 250 μg/ml. The phagocytic ability of these cells was significantly hindered by exposure to each size of the Al-NP at 25 μg/ml for 24 hours, but not by the Al_2O_3-NP. A series of cytokine and nitric oxide assays performed show none of these aluminum nanoparticles are inducing an inflammatory response.

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I am indebted to many of the laboratory technicians and faculty at AFRL especially Lt Carlson, TSgt Jones, Dr Hess and Ms Schrand. I am also thankful for the leadership at AFRL, without Col Riddle, Dr Schlager and Lt Col (s) Johnson's support this collaborative effort would not have been possible.

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Andrew J. Wagner

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List of Symbols

(AM)	Alveolar macrophage
(NP)	Nanoparticles
(Al_2O_3-NP)	Aluminum oxide nanoparticles
(Al-NP)	Aluminum nanoparticles
(MTT)	Tetrazolium salt, 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyl tetrazolium bromide
(SEM)	Scanning Electron Microscopy
(R&D)	Research and Development
(MSDS)	Material Safety Data Sheets
(TSCA)	Toxic Substance Control Act
(EPA)	Environmental Protection Agency
(NIOSH)	National Institute for Occupational Safety and Health
(NSF)	National Science Foundation
(AM)	Alveolar macrophages
(NO)	Nitric Oxide
(TNF-alpha)	Tumor necrosis factor-alpha
(ATP)	Adenosine Triphosphate
(NADH)	Nicotinamide adenine dinucleotide
($FADH_2$)	Flavin adenine dinucleotide
(AFRL)	Air Force Research Lab
(FBS)	Fetal bovine serum
(PBS)	Phosphate buffered saline
(P.I.)	Phagocytosis index
(LPS)	Lipopolysacaride
(ELISA)	Enzyme-Linked ImmunoSorbent Assay
(TEM)	Transmission Electron Microscopy

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IN RAT ALVEOLAR MACROPHAGES

I. Introduction

1.1 Background

Nanotechnology provides a fundamental understanding of phenomena at the nanoscale level in order to create structures and devices that have novel properties and functions. Nanomaterials may benefit the military and society, but currently there is little characterization of the effect of these materials on the environmental or human health. Nanoparticles (NP) and materials are of interest to the Air Force because of their applications in electronics, sensors, munitions and energetic/reactive systems. Metal nanoparticles, such as aluminum, have applications, but only a few studies, none with aluminum, have looked at the toxicological effects of direct exposure to metal nanoparticles. Characterization of potential nanoparticle exposure health impacts is critical, before these particles are fully integrated into industry and the military.

NASA is currently investigating Al nanoparticles to increase the specific impulse of composite propellants in solid rocket fuel (Palazewski, 2002). (Miziolek, 2004) at the Army Research Lab , demonstrated certain Al nanoparticle composites have great promise in explosives research. Further, in 2004 the U.S. Naval Air Warfare Center investigated aluminum nanocomposites as “green” bullet primers and currently the Navy is using a nanocomposite of alumina-titania as wear resistant coatings on propeller shafts (Department of Defense Director, Defense Research and Engineering, 2005) and (Loney, 2004).

1.2 Problem Identification

Although there are numerous applications of nanoparticles, specifically Al nanoparticles, there is little information on their biocompatibility or toxicity. Only a few studies have looked at the toxicological effects of direct exposure to metal nanoparticles, however no studies have been done on aluminum toxicity.

1.3 Research Questions

The purpose of this investigation was to determine the effect of aluminum nanoparticles on Rat Alveolar Macrophages. Specifically, the following questions were addressed:

1. What effects do aluminum nanoparticles have on the viability of *in vitro* rat lung macrophages?

2. What are other exposure effects (morphology, phagocytosis, immune response) of aluminum nanoparticles on *in vitro* rat lung macrophages?

1.4 Research Focus

The focus of this basic research was limited to the *in vitro* exposure of rat alveolar macrophages to various types of Al nanoparticles. The goal was to give insight into inhalation toxicity of these particles in order to explore future modeling and *in vivo* exposure studies to develop recommended work place exposure limits.

1.5 Methodology

In Vitro techniques were used in this research. Alveolar macrophages (AM) were cultured in plastic flasks and exposed to various concentrations of Al_2O_3-NP and Al-NPs for different time periods. The viability, phagocytic ability, inflammatory response, and morphological characteristics of the cells were all observed.

The viability of the AM after exposure to each type of Al nanoparticle at various concentrations was determined by measuring the mitochondrial function of the cell as an indicator of metabolic activity and thus viability. The colorimetric assay evaluates mitochondrial dehydrogenase reduction of Tetrazolium salt, 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyl tetrazolium bromide (MTT) to a blue formazan product (Carmichael *et al.,* 1985).

AM phagocytosis, after exposure to each type of Al nanoparticle, was measured by uptake of 2 μm latex beads, and observed with an Olympus IX71 inverted fluorescent microscope with CytoViva as a light source. A phagocytosis index was determined and compared to cells not exposed. This method was developed from Paine *et al*., 2004.

The inflammatory response of the cells, after exposure to each type of Al NP was observed by determining the amount of inflammatory cytokines and Nitric Oxide produced. *In Vitro* assay kits from Biosource Inc. were used to determine cytokine levels and *in vitro* kits from Promega Inc. were used to determine Nitric Oxide production.

Morphological characteristics of the cells, after exposure to each type of Al-NP, were observed with three different imaging techniques--basic light microscopy with an Olympus CK2-001T microscope, light microscopy with an Olympus IX71 inverted

fluorescent microscope with Cyto Viva as a light source, and Scanning Electron Microscopy (SEM).

1.6 Assumptions/Limitations

(1) Charaterization properties of Al NPs were expected to change after dry powder particles were suspended in deionized water and media.

(2) Cells dosed at the same Al concentration had an equal level of exposure throughout the experiment and the exposure method used was satisfactory.

(3) Despite the lack of evidence as to the effects of aluminum NP on human health, aluminum NPs were treated as possibly toxic to respiratory cells.

(4) NP concentrations were selected from preliminary MTT assay results and used in the functional assay experiments. These concentrations were not based on particle deposition data.

(5) *In Vitro* results only give insight to expected future *in vivo* results

1.7 Implications

Nanoparticles are being integrated into the workplace with little effort to identify potential health and environmental effects. Research like this will help evaluate acceptable exposure limitations and measurements and help define control requirements and future regulations. Identification of possible health and environmental effects is necessary if nanotechnology is to become more common. Early characterization of negative health and environmental effects of these nanoparticles can help avoid previous mistakes such as the wide spread misuse of asbestos and DDT.

1.8 Overview of Document

This document contains five chapters.

Chapter Two: Reviews selected literature on current and future uses of nanoparticles, including: health and safety aspects, and an overview of aluminum toxicity. It also demonstrates that toxicity in alveolar macrophages can be characterized according to cell viability, phagocytosis ability, immune response, and morphology.

Chapter Three: Provides a concise explanation of the methods used to obtain data.

Chapter Four: Presents an analysis of data.

Chapter Five: Draws final conclusions and suggests future research.

II. Literature Review

2.1 Background

This literature review provides a concise discussion on the current and future uses of nanoparticles in society and the current health and safety aspects of these particles. It also includes specific uses of aluminum nanoparticles and a broad overview of the current understanding of aluminum toxicity and its involvement in disease and environmental degradation. This literature review also demonstrates that toxicity in alveolar macrophages can be characterized according to cell viability, phagocytosis ability, immune response, and morphology.

2.2 Nanoparticles (NPs)

NPs range between 1-100nm in size and display useful electrical, thermal, mechanical and imaging properties; however, an accurate characterization of their risk to health and the environment is lacking. As NP applications improve and expand, the risks these particles present should be compared to their potential benefits (Dreher, 2004),(Colvin 2003). The large potential of these particles is seen by the large amounts of funding that the United States and other countries have invested in research and development of nanotechnology.

2.2.1 Current and Future Uses

There are numerous different applications that currently exist for nanotechnology, and it would be impossible to cover all of them. In order to gain insight as into how this technology is growing, a few examples of current nanotechnology and future applications are presented in this section.

In the medical field, NPs are used in drug and vaccine delivery, including intravenous, intramuscular, and subcutaneous injections, and including oral and ocular administration (Kreuter, 1991). NPs allow delivery of drugs that are non-ionic and not water soluble. By encapsulating a drug with an NP hybrid, such as magnesium aluminum, the solubility of a drug can be increased and also provide potential for site direction to target cells. This encapsulation has proven to increase the solubility of some drugs and holds promise for many other types (Tyner *et al*., 2004). Anti-tumor drugs, delivered by NPs, might improve therapeutic response and even allow for monitoring the amount of drug taken in by tumor cells (Kukowska-Latallo *et al*., 2005). Not only are NPs being used to get the drugs to the right place, but they are also being looked at as drugs themselves. Silver NPs have shown to have the ability to attach to the glycoprotein knobs on the HIV-1 virus and prevent the virus from entering cells and replicating (Elechiguerra *et al*., 2005).

Electronics and sensors are some of the most promising areas of research and development (R&D) for NPs. These particles have properties that make them desired as conducting composites. For example, carbon nanotubes can carry more current density than any metal, as high as 1000 times the current density of copper (Kuennen, 2004). Interactive touch screens can be produced with optically transparent conductive coatings of indium tin oxide and antimony tin oxide nanoparticles that are simply painted onto surfaces (Robertson, 2004). These conductive properties provide the ability to produce devices at the nano scale and leading to improvements in sensor technology. Currently there are 200 tiny sensors called Micro-Electronic-Mechanical Systems (MEMS) on the Golden Gate Bridge. They are used to collect data on traffic, wind, and seismic activity. Future applications of these small sensors could be used on bridges and roads all over the

world. The department of Defense has used the word “smart dust” to describe similar sensors that would be the size of a period. These could be used as suggested above, or on the battle field as real time reconnaissance tools (Kuennen, 2004).

NPs are important in improving current materials. Surfaces can be made resistant to abrasion with the addition of aluminum oxide and titanium oxide particle coatings (Dingman, 2005) and nanotechnology can lead to surfaces that are able to shed water (Kuennen, 2004). Metal matrix composites are being explored for improved applications in defense, aerospace and automotive industries. Composites such as carbon, boron and silicon carbide are used to reinforce aluminum and magnesium. Improved properties of these metal composites have allowed them to have many new aerospace applications (Dingman, 2005) and (Robertson, 2004). Work is being done to explore self healing polymers that might possibly lead to “self-healing roads” and numerous other possible applications on high wear areas (Kuennen, 2004).

Nanomaterials are also being explored as a sustainable way to capture energy from the sun. Nanotechnology is now at a point where thin films of organic nanostructures can be made. These thin layers of semiconducting organic materials capture photons from the sun and use them as energy. These organic photovotaics have a lot of promise due to the fact they are cheap and easy to make (Dingman, 2005). Photovoltaic fibers have been developed and demonstrated as a new technology to be used by the military (Department of Defense Director, Defense Research and Engineering, 2005).

We may soon find ourselves in contact with silver, zinc and copper nanoparticles in our everyday lives, due to their anti-microbial characteristics. A good example of this is zinc oxide nanopowders that are being explored for uses in deodorants, dental cleansers,

and diaper creams. Copper oxide nanopowder is also a possible future product as an antimicrobial preservative for wood and food products. (Dingman, 2005).

The importance of nanotechnology is evident due to the global growth of RD in this area. This is seen in the amount of money that governments are allocating to RD. Funding towards RD has increase seven fold from 1997 to 2003 and the United States has been a leader in the pack with $849 million dollars in 2004. Table 2-1 breaks down the major contributors toward RD in nanotechnology over the last eight years. Industry believes that worldwide production in nanotechnology could reach 1 trillion dollars in the next 8 to 12 years, and this would require about 2 million nanotechnology workers (Roco, 2003). President Bush signed the 21st century Nanotechnology Research and Development Act that allocates $3.4 billion dollars to the National Nanotechnology Initiative over the next four years from 2005 thru 2008, Table 2-2 (Kuennen, 2004). Figure 2-1 Illustrates the relationship between R&D investments and the number of articles concerning nanotechnology in the prominent journal Science over the past decade.

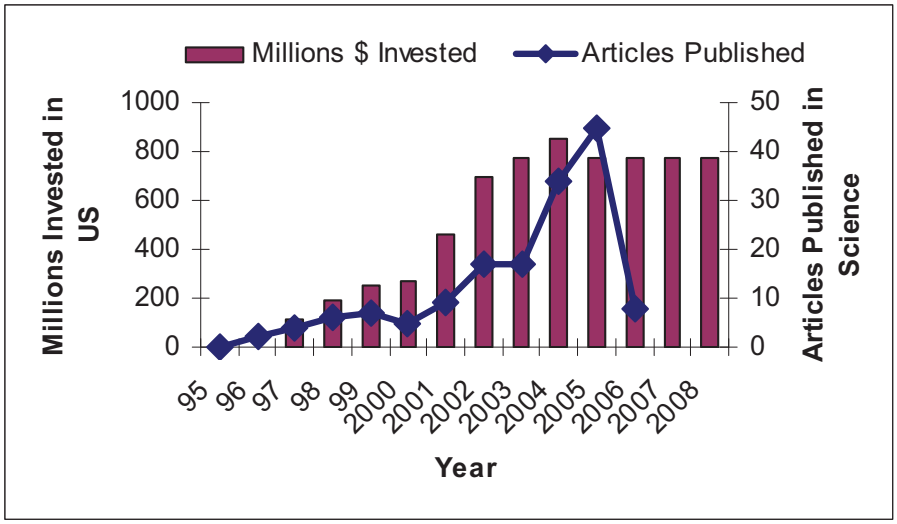


Figure 2-1 Nanotechnology Investments and Articles Published in Science

Blue line indicated the number of articles published in the Journal Science (determined by an on-line search using nanotechnology, nanomaterial, and nanoparticles as key words in the title and abstracts of the articles) (Science, 2006). The bar graph numbers were taken from (Roco, 2003)

Table 2-1 - Research and Development Money Invested in Nanotechnology (Roco, 2003)

Region	1997	1998	1999	2000	2001	2002	2003	2004R
W. Europe	126	151	179	200	~225	~400	~650	
Japan	120	135	157	245	~465	~720	~800	
USA	116	190	255	270	465	697	774	849
Other	70	83	96	110	~380	~550	~800	
Total (% of 1997)	423 100%	559 129%	687 159%	825 191%	1,535 355%	2,367 543%	3,024 700%	

Table 2-2 – Allocation of funds for the 21st century Nanotechnology Research and Development Act (Kuennen, 2004)

Agency	Fiscal 2005	Fiscal 2006	Fiscal 2007	Fiscal 2008
NSF	$385,000,000	$424,000,000	$449,000,000	$476,000,000
DOE	$317,000,000	$347,000,000	$380,000,000	$415,000,000
NASA	$34,100,000	$37,500,000	$40,000,000	$42,300,000
NIST	$68,200,000	$75,000,000	$80,000,000	$84,000,000
EPA	$5,500,000	$6,050,000	$6,400,000	$6,800,000
Total	**$809,800,000**	**$889,500,000**	**$955,400,000**	**$1,024,100,000**

2.2.2 Uses of Aluminum Nanoparticles

Aluminum nanoparticles have been used in drug delivery, improvements in surface coatings, and in improvements of metal alloys. Production of nanopowders such as iron aluminide (FeAl) and iron aluminum carbide ($Fe_3AlC_{0.5}$) show promise in low density, high tensile strength, and corrosive resistant material production. It has been suggested that reducing the size of FeAl to the nanoparticle range will increase the strength of these materials. The uses of $Fe_3AlC_{0.5}$ with FeAl offer the same advantages as Ni_3Al, but at a lower cost (Pithawalla, 2004).

Aluminum nanoparticles are potential ingredients for solid rocket fuels of the future. Aluminum was first introduced to rocket fuel in the early 1960's when Keith Rumbel and Charles Henderson found that adding aluminum increased the specific impulse of a composite propellant and that one of the many conditions for good combustion was a small particle size. Key missiles and boosters that have used aluminum as fuel are the Polaris rockets A1-A3 established 1960-1964, the Minuteman I rockets Stage 1-3 in 1962, the Titan 3 solid-rocket motor in 1965, and the current space shuttle solid-rocket booster in 1981 (Hunley, 1999). In a hypothetical mission to Mars,

Palazewski infers that adding micro sized aluminum particles to solid rocket fuel, at 60% by weight, could increase the thrust of the rocket enough to carry 22% more payload and as much as a 33% increase by adding 70% by weight (Palazewski, 2002). Past usage of aluminum in solid rocket fuel has been in the micrometer size range, but future applications may involve NASA and DoD using nanoscale aluminum nanoparticles as a propellant ingredient (Romano, 2005). The particles NASA is investigating range in size from 20 to 100 nm with a 2 to 3 nm thick oxidized coat (Aluminum oxide Al_2O_3). This new size of particle will allow for increases in fuel density, safety, and exhaust velocity. It will also reduce fuel slosh, leakage, and the overall size of the vehicle (Palaszewski, 2002). Programs have been set in motion to explore the benefits of the metallized gelled rocket propellants by the U.S. Army Missile Command and the NASA Lewis Research Center (Palaszewski, 1997).

Another potential DoD use for these particles is in explosives and artillary. Miziolek, at the US Army Research Lab, looked at Metastable Intermolecular Composites (MICs). These are mixtures of metallic nanopowders that have an exothermic behavior. In an explosive reaction, the amount of energy being released can be dictated by the size of the nanoparticles in the weapon. Three MICs are showing promises and all three contain aluminum nanoparticles: Al/MoO_3, Al/Teflon, and Al/CuO (Miziolek, 2002). Aluminum nanoparticles are being explored by the U.S. Naval Air Warfare Center as a possible replacement for lead primers in artillary. If all tests are successful, this new type of "green" bullet is likely to be used some time next decade (Loney, 2004).

Currently the Navy is using a thermal spray made of a ceramic nanocomposite alumina-titania. This nanomaterial is used to produce wear resistant coatings on

propeller shafts of Mine Countermeasure Ships. With support from Small Buisness Innovative Research this product has been commercialized and is being evaluated for possible use on the rest of the fleet and on through-hull ball valves on submarines (Department of Defense Director, Defense Research and Engineering, 2005).

2.3 Health and Safety

Exposure to nanoparticles will keep increasing due to improvements to the quality of consumer products, medicine, and other technological applications (Dreher, 2004). Assessment of health and environmental risks of nanoparticles, both in industry and society, are lacking. Due to their different characteristics these particles should be considered separate from their macro sized counterparts, in terms of toxicity. As applications improve and expand, the question that needs to be answered is whether the risks these particles have on health and the environment is reasonable compared to their potential benefits to society. (Dreher, 2004) and (Colvin, 2003).

2.3.1 Surface Characteristics

One of the physical properties that make nanoparticles desirable could also increase their toxicity. When the size of these particles decreases, the free surface area of the material at the same volume will increase dramatically. These free surfaces interact with cells. Gupta *et al* in 2004 found that coating superparamagnetic iron oxide nanoparticles, which are toxic to cells, with pullalan resulted in no toxicity and a different endocytosis behavior as compared to uncoated nanoparticles. Yang *et al* in 2005 found that when aluminum nanoparticles are coated with phenanthrene that their pytotoxic effects on root elongation in corn, cucumber, soybean, cabbage, and carrots was reduced

compared to aluminum that was not coated. Renwick concluded from his research that ultra fine carbon black, at a low volume, will impair macrophage phagocytosis due to its high surface area (Renwick *et al*, 2000). These examples are relevant in understanding surface interactions these cells have with the nanoparticles and why the toxicity of these materials should be characterized within a range of different sizes and surface characteristics.

2.3.2 Occupational Exposure

Mulhausen and Domingo in 1998 present a strategy for assessing and managing occupational exposures. As seen in Figure 2-1, goals are set first, then a basic characterization of the potential toxicants is identified in order to establish acceptable level of exposures. Next, these levels are measured over time and more controls are added if exposure is too high, more information is gathered if exposure level is uncertain, or reassessed if exposure levels are acceptable. This cyclic process feeds data back into the basic characterization step (Mulhausen and Domiano, 1998:6-12).

The basic characterization for aluminum is available in numerous locations. Material Safety Data Sheets (MSDS) are good initial source of information, but they are limited and may not be adequate, due to lack of toxicology data (Mulhausen and Domiano, 1998:30). The health effects of excessive exposure to aluminum in general will be addressed in the next section, a list of significant occupational sources of exposure and blood/serum/urine levels is seen below in Table 2-3. MSDSs, the table below, and others sources do not include nanoparticle exposure and health risk information. Overall the characterization of nanoparticles such as aluminum and aluminum oxide compared to their macro sized counterparts is lacking.

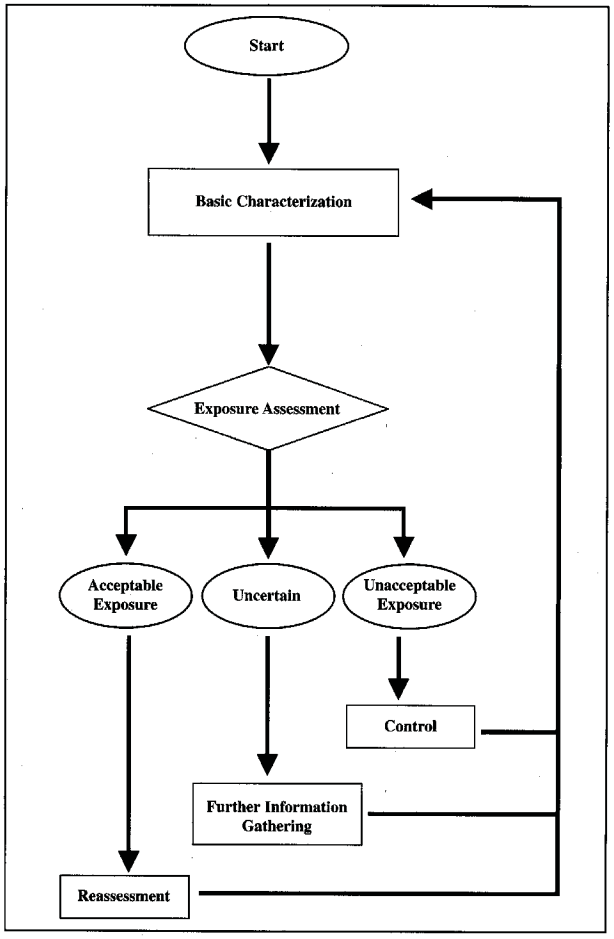


Figure 2-2 - A strategy for assessing and managing occupational exposure (Mulhausen and Domiano, 1998:7)

Table 2-3 - Blood/plasma/serum and urine concentrations of aluminum in workers occupationally exposed and in occupationally nonexposed (Yokel and Golub, 1996, Ch 8: 169).

Exposure (reference)	Blood/plasma/serum			Urine		
	M	Highest	(*n*)	*M*	Highest	(*n*)
Al powder production (Sjögren et al., 1983)	13	171	(20)	37	4230	(19)
Al powder production (Lang & Letzel, 1995)	9	—	(32)	110	—	(32)
Al sulfate production (Sjögren et al., 1983)	9	21	(23)	6	46	(22)
Corundum (Al_2O_3) production (Valentin et al., 1976)	13	164	(110)	39	220	(110)
Cryolite production (Grandjean et al., 1990)	22	46	(8)	100	150	(8)
Electrolytic production (Sjögren et al., 1983)	<5	15	(31)	7	20	(31)
Electrolytic production, potroom 1 (Röllin et al., 1996)	5	—	(12)	67	135 (*M* + 2SD)	(12)
Electrolytic production, potroom 2 (Röllin et al., 1996)	7		(39)	133	260 (*M* + 2SD)	(36)
Grinding (Harwerth et al., 1987)	12	24	(51)	12	37	(48)
Grinding (Elinder & Sjögren, 1990)	—	—		4	18	(14)
Melting and foundry (Elinder & Sjögren, 1990)	<3	<3	(16)	10	44	(16)
Welding (Sjögren et al., 1983)	8	67	(18)	80	2013	(18)
Occupationally nonexposed		<10			<10	

The EPA and the National Science Foundation recognized the following risk assessment issues that still need to be addressed regarding the manufacturing of nanoparticles: exposure assessment, toxicology, extrapolation of toxicology using existing particle and fiber databases, recyclability of materials, and environmental fate, transport, persistence and transformation of nanoparticles (Dreher, 2004). The Institute of Occupational Medicine prepared a report for the Health and Safety Executive in 2004. In this report they looked at nanoparticle occupational hygiene and, in summary, found

there is not enough evidence to say that the exposure of workers from the production of nanoparticles has been adequately assessed (Aitken *et al*., 2004).

2.3.3 Regulations

Concerns with production of nanoparticles include adverse effect on human and environmental health, bioavailability, and transport. The EPA has primarily focused its authority to regulate nanotechnology under the Toxic Substance Control Act (TSCA). This act requires the listing of all chemicals in U.S. commerce, a review of new chemicals before they are introduced, testing the chemicals for their effect on human and environmental health, and establishing reporting, recordkeeping, import and export requirements for chemicals. The Clean Air Act, Clean Water Act, Resource Conservation and Recovery Act, and the National Environmental Policy Act also have some influence and pull on nanotechnology (Hoffman, 2004). These current regulations are in place to control production of new nanotechnologies, but their adequacy is still questioned. This debate will continue until the health and environmental effects of these nanoparticles are fully characterized.

2.3.4 Future Concerns and Funding

The mass production and expanding scope of use of these nanoparticles in society has caused some concern. Asbestos had a great number of uses and a great deal of promise in industry, but the characterization of asbestos in regard to human health was only fully understood recently. In President Bush's January 2005 press release, he estimates that future transactions in asbestos lawsuits will be between $145 and $210 billion (White House Press release 2005). DDT is another example of toxic chemical whose risk to the environment was never fully characterized before its use (Hoffman,

2004). These two examples may be extreme, but they illustrate the value of characterizing these nanoparticle effects on human and environmental health before they are totally integrated into society.

In 2005 the US EPA, National Institute for Occupational Safety and Health (NIOSH), and the National Science Foundation (NSF) awarded $7 Million in grants for research on implications of nanotechnology and manufactured nanomaterials on human health and the environment (EPA, 2005). This is a small fraction compared to $3.4 billion by the 21st century Nanotechnology Research and Development Act seen in Tables 2-1 and 2-2.

2.4 Aluminum Toxicity

Aluminum is the third most abundant element in the lithosphere, behind silicon and oxygen, but is almost non existent in humans and biological systems. Exley explains that biochemical cycles of elements, from the lithosphere into organisms, can give insight into natural selection against these elements by organisms, and creates a strong case that aluminum has been excluded from biochemical processes via evolution (Exley, 2003). If organisms have been excluding uptake of aluminum over evolutionary time there must be some good reasons. The next two sections will address problems involving environmental contamination and known human diseases associated with aluminum in general.

2.4.1 Environmental Contamination

Aluminum toxicity has been discussed at length with regards to decreases in soil and water pH. Figure 2-2 illustrates that the percent of total aluminum and Al^{+3} species will increase as pH decreases. Once pH is below 5.5 toxic effects have been documented

for plants, invertebrates, amphibian larva, and fish. At a pH below 4.5 the dominant species is Al^{+3} and hinders cation exchange and electrolyte balances in organisms.

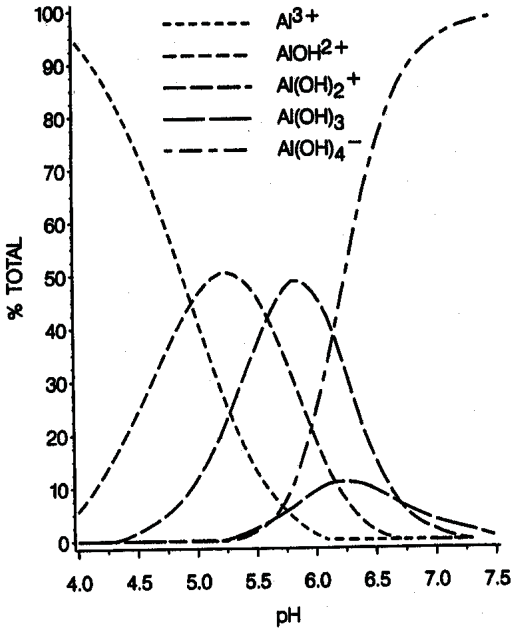


Figure 2-3- Solubility of Aluminum Species (Percent solubility versus pH) (Yokel and Golub, 1996: Ch 1, 20)

Decreases of pH between 6.5 and 5.5 have caused fish to experience asphyxiation. This is due to aluminum's effects on the gills, causing high ventilation rates, low oxygen blood tension, and overall gill damage that include an increase in gill mucus production, and coughing rates (Yokel and Golub, 1996: Ch 3, 50-52). High aluminum concentrations affect ion and calcium regulation and respiratory rates in invertebrates. One study found that mayflies will tend to avoid high concentrations of aluminum in water (Yokel and Golub, 1996: Ch 3, 49-50). Since aluminum has found its way into the food chain by accumulating in plants and invertebrates, birds and mammals both are most likely to be exposed to aluminum via their diets. In birds, aluminum affects egg production, bone structure and embryonic development. In domesticated mammals it has

been found to cause a decrease in food intake, a decrease in weight gain by young animals, and an increase in weight loss by adults (Yokel and Golub, 1996: Ch 3, 52-56).

Aluminum is also a phytotoxin and is considered to be a major limiting factor in crop production (Noble, et al 1988). Again, toxicity is seen when aluminum is leached out of soil via a drop in pH. This is significant since low pH soils make up 30% of land area on the world (Yokel and Golub, 1996: Ch 3, 52-56). Plant effects caused by aluminum are well documented. Noble identified a correlation between the decreased tap root length in soy bean plants and the amount of liable aluminum available to the plant (Noble, *et al.* 1988). Pavan found that aluminum concentrations at 62 to 100 micrograms per gram of leaf in coffee plants causes a decrease in root and shoot growth. He also found that coffee seedling lengths also correlated to aluminum available in the soil (Pavan *et al*., 1982). Root elongation in barley seedlings is strongly correlated with Al^{+3} species, but not with total aluminum (Cameron *et al*., 1986). This again relates back to the pH levels causing Al^{+3} to become the dominant species.

2.4.2 Exposure and Human Disease

Inhalation exposure to aluminum has been linked to numerous physiological problems and a few specific diseases. The Toxicological Profile for Aluminum developed by the U.S. Department of Health and Human Services contains a concise history of research in aluminum occupational exposure. It contains information on symptoms that are seen after exposure via inhalation, oral, and dermal contact. For the purpose of this research, the inhalation exposure effects, along with other current literature, will be reviewed to evaluate the currently known links between aluminum exposure and disease.

2.4.2.1 Fibrosis

Idiopathic Pulmonary Fibrosis as defined by the Merk Manual is the "Chronic inflammation of the alveolar walls with progressive fibrosis, of unknown etiology" (Beers and Berkow, 1999: 635). Fibrosis is the most common reported respiratory effect observed by workers chronically exposed to aluminum dust, powders or fumes. There are a few studies in which male factory workers that were chronically exposed to aluminum flake powder had died as a result of hypertrophy of the right side of the heart due to fibrosis. Most cases of occupational exposure to aluminum involve simultaneous exposure to another particle. In many cases, the other material, such as silica, is found to be the agent that causes the fibrosis. A number of studies have looked at aluminum dust and fume exposure in potrooms and found workers exhibiting symptoms such as wheezing, dyspnea, and impaired lung function, but again since these workers are exposed to a number of other chemicals already considered toxic, respiratory effects of aluminum could not be characterized (Toxicological Profile for Aluminum (update), 1999, 14,23).

2.4.2.2 Hematological Effects

Aluminum exposure has been seen to affect red blood cell numbers, oxygen uptake ability, and clotting ability. Anemia as defined by the Merk Manual is the "Decrease in the numbers of RBCs or hemoglobin content caused by blood loss, deficient erythropoiesis, excessive hemolysis, or a combination of these changes" (Beers and Berkow, 1999: 849). Aluminum causes decreased hemoglobin synthesis in bone marrow cells and erythroleukemia cells resulting in microcrytic hypochromic anemia. Aluminum also appears to affect the amount of intracellular iron available to hemoglobin (Yokel and Golub, 1996: Ch 7, 137-140). In the case of acute occupational exposure, no

hematological effects are seen, but decreases in red blood cell hemoglobin and increased sedimentation rates have been reported on workers that are chronically exposed to aluminum flake powder. In a study on 36 workers that were chronically exposed to aluminum dust, 30 of them had an increase in blood clotting times (Toxicological Profile for Aluminum (update), 1999: 25).

2.4.2.3 Endocrine System Effects

Aluminum exposure has been identified to have certain effects on endocrine organs or the hormones they produce. Post mortem enlargement of the thyroid gland was found in a male factory worker that was chronically exposed to aluminum dust via inhalation (Toxicological Profile for Aluminum (update), 1999: 27). Baydar recognized that there are many similarities between chromium (Cr), essential in glucose metabolism, and aluminum. Urine and plasma samples were taken from diabetic patients and healthy volunteers to determine the Al/Cr ratio. The ratio was high in diabetic patients compared to the healthy controls suggesting that there may be a mechanism where aluminum levels might induce diabetes (Baydar *et al*., 1997). Another study was done on the production of testosterone after aluminum exposure. With the use of rats, it was determined that inhalation of AlCl will increase nitric oxide synthesis and this will inhibited testosterone production (Guo *et al*., 2001). Chih-Hung in 2003 also suggests that the nitric oxide produced is responsible for allowing aluminum to cross the blood-testis barrier and accumulate in the testis (Chih-Hung *et a*l, 2004). This may have significance in aluminum crossing the blood-brain barrier and causing neurological effects.

2.4.2.4 Neurological Effects

Aluminum welders, chronically exposed to aluminum fumes, were evaluated on neurobehavioral performance, and found to have no alterations, but in those workers, there was a correlation between urinary levels of aluminum and memory test performance. There was also a correlation between plasma aluminum levels and visual reaction time tests. Other studies found that aluminum foundry workers and welders had alterations in eye hand coordination, memory, and sub clinical tremors (Toxicological Profile for Aluminum (update), 1999: 29-30).

Alzheimer's Disease as defined by Merck is " A progressive, inexorable loss of cognitive function associated with an excessive number of senile plaques in the cerebral cortex and subcortical gray matter, which also contains beta-amyloid and neurofibrillary tangles consisting of tau protein" (Beers and Berkow, 1999: 1395-1396). In 1994, Shin determined that aluminum binds to these tau proteins and causes them to aggregate and to not go through proteolysis. This results in aluminum modulating tau plaques and tangle formation as described in the definition of Alzheimer's disease (Shin *et al*., 1994). Aluminum-induced tangles tend to happen in the neurons of the spinal cord, cerebellum, brain stem, and in long term studies, they were found in cortical areas and the hippocampus of the brain (Itzhaki, 1994). In 1996, a control study was completed that indicated no relation between occupational exposure due to inhalation of aluminum dust or fumes to Alzheimer's disease, resulting in more emphasis being placed on oral exposure of aluminum and Alzheimer's disease (Toxicological Profile for Aluminum (update), 1999: 30).

2.4.2.5 Musculoskeletal Effects

A few occupational exposure studies have been done on aluminum exposure and musculoskeletal effects. One study identified a female that exhibited joint pain after chronic inhalation exposure to alumnite residue, and another reported chronic inhalation exposure to aluminum powders by two male factory workers resulted in clubbed fingers (Toxicological Profile for Aluminum (update), 1999: 26). Merck defines clubbed fingers as the "enlargement of the terminal digital phalanges with loss of nail bed angle". This condition is seen in a variety of cases including numerous pulmonary diseases (Beers and Berkow, 1999: 520). Most musculoskeletal effects are seen on dialysis patients that are subjected to high intravenous aluminum concentrations. High aluminum levels retard bone remodeling by slowing both osteoclasts and osteoblasts, and resulting in osteomalacia and adynamic bone disease (Yokel and Golub, 1996: Ch 7, 134-137).

2.4.2.6 Cancer

In 1977 a case-control study was done in Quebec, Canada to determine if chronic aluminum exposure via inhalation was the cause for an increase in the number of bladder cancers found in aluminum production plants. The study looked at a 10 year period and 488 cases of which 96 were identified as aluminum production plant employees. The study found that the risk of cancer increased with an increase in estimated exposure to tar and PAHs. The International Agency for Research on Cancer (IARC) made the decision that not enough evidence was available to support a theory that aluminum increases the risk of bladder cancer. They felt that cancer risk in the aluminum production industry is probably due to known carcinogens already in the plants (Toxicological Profile for Aluminum (update), 1999: 31).

2.5 Macrophages

Currently, macrophages are studied *in vitro* in almost all fields of biology. They are found in various tissues, but they all arise in bone marrow then circulate in blood where they eventually migrate to specific sites. They can be obtained from blood, lungs, spleen, liver, and the peritoneal cavity (Jakoby and Pastan, 1976: 494-507). Alveolar macrophages (AM) are found in the alveolar sacs deep with in the lungs where they are the first line of immunological defense from inhaled particles and serve as a good model to understand how inhaled particles can adversely affect health (Kleinman *et al*., 2003). Many studies have used macrophages and their responses to characterize potential toxins. Yang, *et al.,* (2000) found that diesel exhaust particles suppress macrophage function. Also, Ortega *et al.* (1992) performed an experiment that illustrated AM decline in phagocytosis ability after exposure to cigarette smoke and Furukana *et al*. (2002) characterized the effects of exposure of AM to photocopier toner. These examples, along with many others, have used AM to characterized toxicity of particles that are larger that the nanoparticle size range. Renwick *et al*. (2001) and (2004) explored the differences in toxicity of carbon black and titanium oxide at the nano and macro sizes ranges, showing increased toxicity of nanoparticles compared to macro sized particles. Guang, *et al.,* (2005) characterized the cytotoxicity of carbon nanomaterials such as single–walled nanotubes, multi-walled nanotubes and fullerene on AM.

2.5.1 Macrophage Role In Immunity

These immune cells are responsible for regulating inflammation in the healing process. They respond to chemotactic factors produced by inflammatory tissue by the releasing molecules that regulate the activity of connective tissue. They also produce

angiogenesis factors that stimulate new blood vessel growth. In studies where the number of macrophages was reduced, wounds healed poorly (Paul, 1989, Ch 5:100).

These cells are the first line of defense in an infection and rapidly move into position at infected sites. They release mediators that stimulate an acute phase response and they activate T lymphocytes (Paul, 1989, Ch 5:100). T cells will, in turn, activate macrophages to attack and destroy many types of infectious agents (Paul, 1989, Ch 1:10-14).

2.5.2 Phagocytosis

Phagocytosis is the process that cells go through to take in large quantities of materials that cannot be transported across cell membranes. One of the main functions of macrophages in the immune system is to phagocytose foreign particles. Alveolar macrophages protect the lungs by phagocytosing viable and nonviable particles in the alveolar spaces deep within the lungs. Epidemiological studies indicate correlations between moderate particle concentrations in the air and acute effects such as mortality in heart, lung disease, and chronic lung morbidity (Lundborg *et al*., 2001).

Particles that are not phagocytosed can cause an increase in infection and lung damage (Lundborg *et al*., 2001). Because of this, it is important to identify particles that are taken in by AM that decrease phagocytosis ability. Lundborg *et al* in 2001 showed that fine (macro) and ultrafine (nano) carbon particles exposed to AM at a concentration of 0.2 micrograms/ 10^6 AMs, similar to urban areas, resulted in high particle concentrations outside the cells and a decrease in AM phagocytosis . Renwick *et al* in 2000 looked at carbon black and titanium oxide in the fine and ultrafine sizes ranges. Neither of the compounds was directly toxic to the cells, but a significant reduction in the

ability of macrophages to phagocytose other particles was seen. Ultrafine particles impaired phagocytosis at lower doses, compared to their macro sized counterparts. Guang in 2005, observed that not only do nanoparticles decrease phagocytosis more that macro sized particles, but some particles made of the same material will affect phagocytosis in AM differently, due to different surface characteristics. Single walled carbon nanotubes were shown to impair phagocytosis more that other carbon nanoparticles such as multi-walled nanotubes and fullerene (Guang *et al*., 2005)

Renwick's research on titanium and carbon black found that when particles occupied six percent of the AM volume, clearance of other particles was hindered. When particles occupied sixty percent of the AM volume, clearance of other particles was stopped. They also inferred that ultrafine particle toxicity is more likely to induce inflammatory responses. Fine titanium oxide (250 micrometers), at a nine percent loading in AM, caused a 2-fold increase in detention time of foreign particles, where ultrafine titanium oxide (20 nanometers) at a 2.5 percent loading in AM caused an 8 fold increase in detention time of foreign particles. This significant difference suggests that the mass or volume of a load in an AM is not the determining factor in toxicity, but instead the surface area of the particles is (Renwick, *et al*., 2000).

In these phagocytosis studies, Renwick, Lundborg, and Guang dosed macrophages with specific particles at nano and micrometer sizes and observed phagocytosis ability by adding small latex beads. They observed how well their macrophages phagaocytosed these beads after exposure. In this research, the same type of manipulation was used to determine phagocytic ability of AM exposed to different aluminum nanoparticles.

2.5.3 Nitric Oxide (NO) and Cytokines Production

The ability of these cells to induce an innate immune response after exposure has been measured with cytokine assays (Driscoll, *et al.,* 1990). Cytokine, along with nitric oxide levels, have been used to characterize asthma, chronic obstructive pulmonary disease (COPD), bronchiectasis, and acute respiratory distress syndrome (ARDS) (Chung, 2001, Kobayashi et al., 2005).

Kobayashi explained that ARDS, which has 50-70% mortality, is linked to elevated levels of NO_2, NO_3, and cytokines like Interleukin 6 (IL-6), and Interleukin 8 (IL-8). These elevated conditions could be important in acute lung injury. Nitric oxide synthase has been shown to be elevated in asthma, bronchiectasis, and other lung abnormalities. Kobayashi also points out that alveolar macrophages are the main source of NO and cytokines in ARDS patients and that a long term exposure may be toxic or "cytostatic" to those macrophages and other pulmonary cells in close proximity. These elevated levels result in inflammation, and, sustained inflammation was linked to mortality (Kobayashi, *et al*., 2005). NO has many applications in cell physiology. Depending on the target, it can be a neurotoxin, a vasodilator, a neurotransmitter, or it may regulate the binding of oxygen to hemoglobin. It is also linked to cell survival by blocking proteins involved in apoptosis, and to cell death by increasing peroxynitrate species that block cell respiration (Bossy-Wetzel, *et al*., 2003). NO has a pathogenic role in diseases in the central nervous system, including ischemia, brain stroke, epilepsy, and Parkinson's disease (Bashkatova, *et al*., 1998). AMs also produce NO to be used for antimicrobial defense (Jorens, *et al*., 1995), and to regulating cytotoxicity (Paul, 1989, Ch 28: 772). Increase of NO production has been seen in rat brain tissue after exposure to aluminum and this is significant since high aluminum levels in body tissues have been

linked to Alzheimer's disease (Campbell, *et al*., 2000). High aluminum exposure levels leading to increase NO levels, have also been linked to suppression of testosterone in mice (Guo, *et al*., 2004). Bossy-Wetzel et al. (2003) also noticed that elevated NO concentrations were necessary in brown adipose tissue to stimulate increased mitochondrial efficiency in animals that are hibernating. This process, known as mitochondrial biogenesis, occurs when NO concentrations are slightly elevated, but not high enough for NO to out compete oxygen during mitochondrial respiration (Bossy-Wetzel *et al*., 2003).

Chung observed that COPD, which is a chronic obstruction of expiratory flow that is seen in chronic bronchitis and emphysema, also has links to increased cytokine production. Macrophages in general are the key source to what Chung calls Proinflammatory cytokines. These include Interlukine-1 (IL-1) IL-6, tumor necrosis factor-alpha (TNF-alpha) and colony stimulating factors (CSFs). IL-1, TNF and IL-6 are more specifically called endogenous pyrons that act to increase leukocytosis, release of neutrophiles from bone marrow, increase production of other cytokines, increase production of adhesive molecules, intercellular adhesion molecules and vascular adhesion molecules, and an increase of fibronectin and collagen (Chung, 2001). These cytokines are essential in describing an immune response. Inflammation during the immune response to an exposure is a critical area that needs to be characterized.

2.6 Cell Viability and MTT

Tetrazolium salt, 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyl tetrazolium bromide (MTT), is used to evaluate cell viability. This assay is colorimetric and evaluates how well mitochondrial dehydrogenase of viable cells can reduce MTT to a blue formazan

product (Carmichael et al., 1985). The amount of blue product is measured via a spectrophotometer and is used to determine mitochondrial function and viability of a cell.

2.6.1 Cell Metabolism

Adenosine triphosphate (ATP) is the primary energy molecule of all cells. In eukaryotes ATP is produced through respiration in mitochondria. The three stages of cellular respiration are seen in Figure 2-3. Nutrients such as sugar, fats and some amino acids are used to produce ATP and acetyl groups (two carbon molecules). These acetyl groups then enter the mytosol of the mitochondria and are enzymatically oxidized by the citric acid cycle to carbon dioxide and two types of electron carrier molecules, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). These electron carriers enter a third stage on the inner membrane of the mitochondria. They release an electron down a chain of molecules called the respiratory chain to an oxygen molecule that serves as the final electron acceptor and is reduced to form water. This allows hydrogen ions (H^+) from NADH and $FADH_2$ to be pumped into the intermembrane space. These H^+ ions produce a concentration gradient between the intermembrane space and the matrix of the mitochondria. The H^+ ions will then flow back into the matrix through an integral protein in the inner membrane called ATP Synthase. This flow produces the energy needed to convert ADP into ATP (Lehninger, Nelson, and Cox, 1993: 446-447).

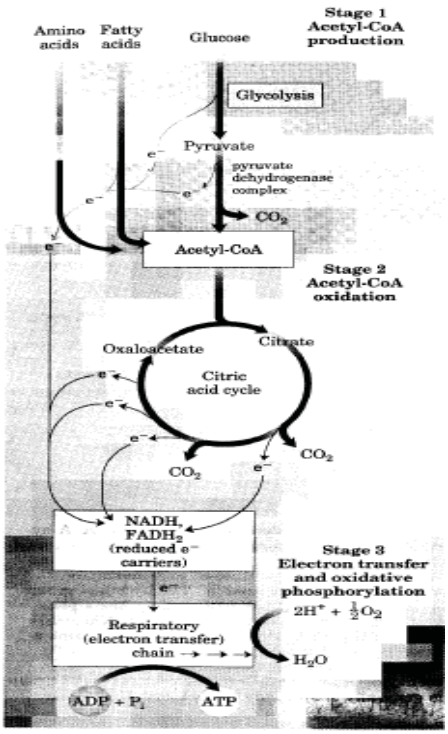


Figure 2-4 - Catabolism of proteins, fats and carbohydrates in eukaryotic cells (Lehninger, Nelson, and Cox, 1993: 446-447).

2.6.2 Mechanism of MTT Assay

MTT is reduced to a blue color if normal metabolic function is maintained by a cell. In order to form this blue color, MTT requires an enzyme located in the respiratory chain called succinate-dehydrogenase. If NADH is available, and in normal metabolic conditions it will be, then MTT will be reduced by succinate-dehydrogenase and the blue foraman product will appear. The amount of blue color produced is proportional to the number and viability of cells and is an indirect measure of cytotoxicity, mitochondrial function, and cell viability (DelRaso, 1996).

2.6.3 MTT Assay Use

In 1985, mitochondria were first being used as a tool to assess chemo-sensitivity of cells via MMT assays. Charmicheal *et al* in 1985 looked at tumor cell mitochondria to see if they had the ability to reduce MMT to its blue color. This assay has been used by the Air Force Research Lab (AFRL) to describe the cellular toxicity of hydrazine in primary rat hepatocytes (Hussain *et al*., 2002). Hydrazine (HzN) is a propellant used by the USAF in rocket fuel. This research is of interest because it was done on a fuel component and accepted and performed at AFRL. The MTT assay was used by Guang to assess the toxicity of single-wall, multi-wall nanotubes and fullerene on alveolar macrophages (Guang et al., 2005), and by Renwick to understand the viability of alveolar macrophages exposed to carbon black and titanium dioxide nanoparticles and micro sized particles (Renwick et al., 2000). These papers illustrate that the MTT assay has been accepted to characterize cell viability of alveolar macrophage cells exposed to various nanoparticles.

III. Methodology

3.1 Introduction

The goal of this research was to characterize the effects of different aluminum nanoparticles on rat alveolar macrophages at different concentrations. This characterization was carried out by *in vitro* methods that used MTT to determine the viability of these cells after exposure and a series of functional assays to determine other effects on the cells such as immune response, ability to phagocytose particles, and morphological changes

Every experiment contained two preliminary steps, cell culture preparation, and exposure of cells to aluminum nanoparticles. These steps are explained in detail, prior to the method explanation of each separate experiment: MTT 24 hr end point, MTT 48, 96, 122 hour end point, Phagocytosis Index determination, Nitric Oxide production, Cytokine production (TNF-a, MIP-2), and morphological changes via Scanning Electron Microscopy (SEM). This methodology will describe assumptions, the lab equipment and nanoparticles, and details of the methods for each of the eight experiments used to gather data.

3.2 Assumptions

Several key assumptions were made at the beginning of the laboratory experiments.

(1) Characterization properties of Al nanoparticles were expected to change after dry powder particles were suspended in deionized water and media.

(2) Cells dosed at the same Al concentration had an equal level of exposure through out the experiment and the exposure method used was satisfactory.

(3) Despite the lack of evidence as to the effects of aluminum nanoparticles on human health, aluminum nanoparticles were treated as possibly toxic to respiratory cells.

(4) Nanoparticle concentrations were selected from preliminary MTT assay results and used in the functional assay experiments. These concentrations were not based on particle deposition data.

(5) *In Vitro* results only give insight to *in vivo* exposure results

3.3 Cell Line

The NR8383 line was obtained from the American Type Culture Collection (ATCC). The cells were established from normal rat alveolar macrophages obtained by lung lavage and isolated on August 3, 1983. ATCC product description indicates these cells exhibit characteristics of macrophages, and they are homogenous and highly responsive alveolar macrophages which can be used in vitro to study macrophage related activity.

3.4 Cell Culture

The cells were maintained in Ham's nutrient mixture F-12 media (Kaighn's modified) and supplemented with 20% fetal bovine serum (FBS), received from American Type Culture Collection (ATCC, Manassas, USA). 1% penicillin and streptomycin, purchased from Sigma Chemical Company (St. Louis, MO), was added to the media mixture. For morphological studies and assays the cells were seeded in 6 or 24-wells plates and on slides at concentrations ranging between $2.5x10^5$ to $1x10^6$ cells per ml. The cells were incubated in a humidified incubator at 35°C and 5% CO_2 atmosphere

on a substrate of rat tail collagen. They were cultured to desired cell density prior to dosing. Details on cell culture for each assay are given in future sections.

3.4.1 Trypsin

Throughout the process of maintaining adequate conditions for cell growth and harvesting cells for assays, the cells were removed from the collagen matrix with trypsin (Appendix A).

3.4.2 Collagen Substrate

Alveolar macrophages require substrate attachment while growing and developing *in vitro*. A substrate was created with the use of rat tail collagen and 1% phosphate buffered saline (PBS). One ml of collagen stock solution (25mg/ml) was mixed with 250 ml PBS. Depending on the container, a small amount of collagen/PBS solution was added to the wells, flasks, and slides. 1 ml per well for six-well plates and two-well slides, 0.5 mL per well in 24-well plates, and 5mL in each flask. Once the collagen was added, the containers were placed under UV light for two to four hours. Then the solutions were removed and the containers sat under UV an additional 2-3 hours to allow drying and to prevent microbial contamination. The containers were then wrapped in plastic and refrigerated.

3.4.3 Cell Count

In order to measure and compare results from many of these assays, the number of cells/ml of media had to be known. This was determined by using 10 µl of cells in media at an unknown, usually a very high, concentration and adding this volume to a hemocytometer. This slide contains numerous grids that are used to help count cells. The cells located in the grids on each corner of the slide are counted, and the average number of cells in each grid is determined to be the number of cells per 10 µl and, by

multiplying that number by 10,000, the number of cells per mL is determined. The total umber of cells in a sample can be determined by multiplying the cells/mL by the number of mL in the sample. The concentration can be adjusted down by diluting the sample, using the following equation $C_{initial} \times V_{initial} = C_{desired} \times V_{desired.}$

3.5 Spectromax 190

The Spectromax 190 spectrophotometer was used in the data analysis during the MTT assays, Nitric oxide assays, and cytokine assays. The spectrophotometer measured emitted light at certain wavelengths for each assay. The SOFTmax Pro software was used to gather and present the data electronically.

3.6 Nanoparticles

The test materials, Al_2O_3-NPs (aluminum oxide nanoparticles 30 nm, 40 nm in diameter) and Al-NPs (aluminum nanoparticles 50 nm, 80 nm, 120 nm in diameter and a 1-2 nm thick oxide coat). were supplied by Dr Karl Martin at Nanotechnologies Inc., Austin, TX. Aluminum nanoparticles, in dry powder form, were suspended in de-ionized water to make stock solutions (10mg/ml). Prior to each use, stock solutions were sonicated for 20 seconds to reduce agglomeration of particles. Agglomeration was characterized with the Olympus IX71 inverted fluorescent microscope with CytoViva as a light source (Appendix G). Dilutions were made in cell culture media from stock solutions and mixed by inverting the container 5 times and vortexing for 5 seconds. The pH values ranged between 7.1-7.8, and were verified with each dose range for each particle size. The different concentrations of nanoparticle suspensions were added to macrophages cultured in 6 and 24 well cultured plates or chambered cultured slides. Details for each assay on aluminum concentrations (dose) are given in future sections.

The Tables, 3-1 and 3-2, categorizes nanoparticles by source, size, assay use, and dosing range.

Table 3-1 Nanoparticles Used

Chemical	Size (diameter)	Source	Assay Use
Al_2O_3	30 nm	Nanotechnologies Inc.	MTT, NO, TNF-alpha, MIP-2, P.I.
Al_2O_3	40 nm	Nanotechnologies Inc	MTT, NO, TNF-alpha, MIP-2, P.I.
Al	50 nm	Nanotechnologies Inc	MTT, NO, TNF-alpha, MIP-2, P.I.
Al	80 nm	Nanotechnologies Inc	MTT, NO, TNF-alpha, MIP-2, P.I.
Al	120 nm	Nanotechnologies Inc	MTT, NO, TNF-alpha, MIP-2, P.I.
Ag	25 nm	Nanotechnologies Inc	MTT positive control

Table 3-2

Nanoparticle Dosing (0, 25,100, 250 μg/mL media)(FBS Fetal Bovine Serum)

Particle	0 μg/mL	25μg/mL	100 μg/mL	250 μg/mL
Al_2O_3 30nm	5 mL 20 % FBS	5 mL of 20 % FBS + .0125mL Al_2O_3 30nm	5 mL of 20 % FBS + .05 mL Al_2O_3 30nm	4.875 mL of 20 % FBS +.125 mL Al_2O_3 30nm
Al_2O_3 40nm	5 mL 20 % FBS	5 mL of 20 % FBS + .0125mL Al_2O_3 40nm	5 mL of 20 % FBS + .05 mL Al_2O_3 40nm	4.875 mL of 20 % FBS +.125 mL Al_2O_3 40nm
Al 50nm	5 mL 20 % FBS	5 mL of 20 % FBS + .0125mL Al 50nm	5 mL of 20 % FBS + .05 mL Al 50nm	4.875 mL of 20 % FBS +.125 mL Al 50nm
Al 80nm	5 mL 20 % FBS	5 mL of 20 % FBS + .0125mL Al 80nm	5 mL of 20 % FBS + .05 mL Al 80nm	4.875 mL of 20 % FBS +.125 mL Al 80nm
Al 120nm	5 mL 20 % FBS	5 mL of 20 % FBS + .0125mL Al 120nm	5 mL of 20 % FBS + .05 mL Al 120nm	4.875 mL of 20 % FBS +.125 mL Al 120nm

3.7 MTT Assay

The MTT assay was used to establish alveolar macrophage viability before and after exposure to Al nanoparticles. MTT was assessed by seeding AM on collagen coated 24 well plates at 5 x 10^5 AM/ml in 20% FBS media for 48 hours prior to desired

assay time. 24 hours prior to assay time the stock solution of nanoparticles were sonicated for 20 seconds, and suspend in 20% FBS media to attain a desired nanoparticle media concentration. Media was removed from each well used during the seeding process and replaced with nanoparticle/media solutions and incubated at 37° C . For a 24 hr end point the MTT assay was completed the next day, for end points longer than 24 hours the MTT assay was completed later, and the media/nanoparticle solutions were replaced with pure 20% FBS media at 24 hrs. Assay was performed 48, 96 or 144 hours after initial exposure to the particles. At the desired endpoint, 100 µl of MTT stock was added directly to each well. Plates were incubated at 37° C for 30 minutes at which time a purple color will developed. The solution was re-suspended and transferred into tubes and centrifuged for 2 minutes at 2,000 x g. The supernatant was discarded, 0.5 ml of 70% isopropanol was added to each pellet and it was vortex well to obtain homogeneous staining. 200 µl of the sample was transferred into wells of a 96 well plate and read on a Spectromax 190 spectrophotometer at wavelengths from 570 nm to 630 nm. The percent reduction of MTT was compared to the 0 control (cells not exposed to nanoparticles) which represented 100% MTT reduction.

Each experiment consisted of collecting data for each nanoparticle size, at each separate concentration, in triplicate. Each experiment was then replicated a minimum of three times. Table 3-3 explains the number samples taken and the number of experiments completed. A detailed explanation of the procedure is in Appendix B.

Table 3-3 MTT Experiments

End Point	0 control	Ag 25nm control 25 μg/ml	Ag 25nm control 75 μg /ml	Al 30nm	Al 40 nm	Al 50 nm	Al 80nm	Al 120 nm
24 hr	S/E = 3 E = 3 TDP = 9	S/E = 2 E = 3 TDP = 6	S/E = 2 E = 3 TDP = 6	S/E = 3 E = 3** TDP = 45	S/E = 3 E = 3** TDP = 45	S/E = 3 E = 3* TDP = 27	S/E = 3 E = 3* TDP = 27	S/E = 3 E = 3* TDP = 27
48 hr	S/E = 3 E = 3 TDP = 9	S/E = 1 E = 3 TDP =3	S/E = 1 E = 3 TDP = 3	S/E = 3 E = 3* TDP = 27	S/E = 3 E = 3* TDP = 27	No Long Term Data	No Long Term Data	No Long Term Data
96 hr	S/E = 3 E = 3 TDP = 9	S/E = 1 E = 3 TDP = 3	S/E = 1 E = 3 TDP = 3	S/E = 3 E = 3* TDP = 27	S/E = 3 E = 3* TDP = 27	No Long Term Data	No Long Term Data	No Long Term Data
144 hr	S/E = 3 E = 3 TDP = 9	S/E = 1 E = 3 TDP = 3	S/E = 1 E = 3 TDP = 3	S/E = 3 E = 3* TDP = 27	S/E = 3 E = 3* TDP = 27	No Long Term Data	No Long Term Data	No Long Term Data

E = Number of experiments; S/E = Number of samples taken in each experiment;

* Cells were dosed at 3 different concentrations of Al (25,100 and 250 μg /ml);

** Cells were dosed at 5 different concentrations of Al (25, 50, 100, 250 and 500 μg /ml);

TDP = Total Data Points used

3.8 Phagocytosis Assay

This *in vitro* assay was used to determine the effects of aluminum nanomaterials on the phagocytic function of alveolar macrophages. Cells were seeded on chambered microscopic slides at 250,000 cells/ml media for 2 to 4 hours. The media was then replaced with a nanoparticle/media mixture at 25 μg/ml for all Al nanoparticle sizes (30, 40, 50, 80, 120 nm) and 5 μg/ml for Al 50, 80, and 120 nm. After 24 hours exposure, the media was replaced with a 2 μm latex bead solution for 6 hours at a 10 to 1 ratio (10 beads per cell). After 6 hours the cells were washed twice with warm media and placed on the Olympus IX71 inverted fluorescent microscope with CytoViva as a light source.

The cells in each field of view were counted along with the number of beads in each cell. 100 cells were counted on each slide and at least 3 slides were counted for each nanoparticle at each concentration. A phagocytosis index (P.I.) was determined by multiplying the average number of beads taken in by positive cells by the percent of macrophages that were positive. Table 3-4 explains the number of experiments that were completed and number of cells counted. A draft for the standard operating procedure for the assay is found in Appendix C.

Table 3-4 Phagocytosis Experiments

Al (μg/ml)	0 control no Al	Al 30 nm	Al 40 nm	Al 50 nm	Al 80 nm	Al 120 nm
25	N/A	CC/E = 100 E = 3 TCC = 300	CC/E = 100 E = 3 TCC = 300	CC/E = 100 E = 3 TCC = 300	CC/E = 100 E = 3 TCC = 300	CC/E = 100 E = 3 TCC = 300
5	N/A	N/A	N/A	CC/E = 100 E = 4 TCC = 400	CC/E = 100 E = 4 TCC = 400	CC/E = 100 E = 4 TCC = 400
0	CC/E = 100 E = 3 TCC = 300	N/A	N/A	N/A	N/A	N/A
Total	300	300	300	700	700	700
					Total	3,000 cells

CC/E = Cells counted per experiment; E = Number of experiments; TCC = Total cells counted

3.9 Nitric Oxide Assay

The nitric oxide assay characterized the effects of Al nanoparticles on an inflammatory response. AM were seeded on collagen-coated 6 well plates with 5×10^5 AM/ml in 20% FBS media for 48 hours. At 24 hours prior to supernatant collection the stock solution of nanoparticles were sonicate for 20 seconds, and suspended in 20% FBS media to obtain a desired nanoparticle media concentrations. The media was replaced with the nanoparticle/media solutions. The wells were incubated for 24 hours and at least

one well was dosed in each experiment with Lipopolysacaride (LPS) as a positive control. In each experiment an extra well was set up so a post treatment cell count could be accomplished by washing cells off their collagen substrate and counting them microscopically on a hemacytometer. Media and cells from remaining wells were collected and placed into tubes and sonicated for 5 seconds to break open cells. Cells were centrifuged at 12,000 g for 8 minutes and supernatants were collected to measure nitric oxide as directed by the manufacturer (Promega). A detailed explanation of the procedure can be found in Appendix D.

Table 3-5. Nitric Oxide Experiments

Exposure	0 control	LPS control	Al 30 nm	Al 40 nm	Al 50 nm	Al 80 nm	Al 120 nm
0 μg/ml	S/E = 2 E = 3	N/A	N/A	N/A	N/A	N/A	N/A
25 μg/ml	N/A	N/A	S/E = 2 E = 2	S/E = 2 E = 2	S/E = 2 E = 2	S/E = 2 E = 2	S/E = 2 E = 2
100 μg/ml	N/A	N/A	S/E = 2 E = 2	S/E = 2 E = 2	S/E = 2 E = 2	S/E = 2 E = 2	S/E = 2 E = 2
250 μg/ml	N/A	N/A	S/E = 2 E = 2	S/E = 2 E = 2	S/E = 2 E = 2	S/E = 2 E = 2	S/E = 2 E = 2
10 nmoles	N/A	S/E = 3 E = 2	N/A	N/A	N/A	N/A	N/A
100 nmoles	N/A	S/E = 2 E = 1	N/A	N/A	N/A	N/A	N/A

3.10 Cytokine Assay

The MIP-2 and TNF-α assays were used to characterize effects of Al nanoparticles on an inflammatory response. AM were seeded on collagen-coated 6-well plates with 5 x 10^5 AM/ml in 20% FBS media for 48 hours. At 24 hours prior to supernatant collection the stock solution of nanoparticles were sonicate for 20 seconds, and suspended in 20% FBS media to obtain a desired nanoparticle media concentrations.

The media was replaced with the nanoparticle/media solutions. The wells were incubated for 24 hours and at least one well was dosed in each experiment with Lipopolysacharide (LPS) as a positive control. In each experiment an extra well was set up so a post treatment cell count could be accomplished by washing cells off their collagen substrate and counting them microscopically on a hemacytometer. Supernatant media from remaining wells were collected and placed into tubes and frozen at -20 degree Celsius for future use. Cells were centrifuged at 13,000 g for 5 minutes and dilutions were made and the cytokine assays were performed as directed by the manufacturer (Biosource). Table 3-5 explains the number samples taken and the number of experiments completed. A detailed explanation of both procedures can be found in Appendix E and F.

Table 3-5 Cytokine assay (TNF-alpha and MIP-2) Experiments

Exposure	0 control	Al 30 nm	Al 40 nm	Al 50 nm	Al 80 nm	Al 120 nm
0 μg/ml	S/E = 3 E = 4	N/A	N/A	N/A	N/A	N/A
25 μg/ml	N/A	S/E = 3 E = 4	S/E = 3 E = 4	S/E = 3 E = 4	S/E = 3 E = 4	S/E = 3 E = 4
100 μg/ml	N/A	S/E = 3 E = 4	S/E = 3 E = 4	S/E = 3 E = 4	S/E = 3 E = 4	S/E = 3 E = 4

S/E = number of samples in each experimental run, E = number of experiments completed

3.11 Scanning Electron Micrograph (SEM)

SEM was used to determine surface morphology changes associated with the exposure of alveolar macrophages to Al nanoparticles. Protocol detail are shown in Appendix K

3.12 Statistical Analysis

All the experiments were done in triplicate and the results were presented as mean ± standard deviation. The experimental data was analyzed by ANOVA using Sigma Stat. and statistical significance was accepted at a level of $p < 0.05$.

IV. Data Description and Analysis

4.1 Introduction

This Chapter presents the data gathered from the methodologies described in Chapter 3. The chapter includes, in this order; 24 hour exposure MTT data of AM to Al_2O_3-NPs ; 48, 96, 144 hour exposure MTT data of AM to Al_2O_3-NPs; 24 hour exposure MTT data of AM to Al-NP; phagocytosis data after 24 hour exposure to Al_2O_3-NP and Al-NP; Immune response of AM via the production of Nitric Oxide, TNF-alpha, and MIP-2 after 24 hour exposure to Al_2O_3-NP and Al-NP; and various images of the cells using light microscopy, CytoViva, ESEM, and SEM.

4.2 Alveolar Macrophage Viability Data

The MTT assay measured the viability of the cells. The colorimetric test evaluates viable cell mitochondrial dehydrogenase reduction of MTT to a blue formazan product (Carmichael et al., 1985). Blue formazan, measured via a spectrophotometer, was used to determine mitochondrial function and cell viability. Percent reduction of MTT by cells exposed to Al nanoparticles was compared to cells that were not exposed to Al nanoparticles (0 controls). In Figures 4-1 to 4-3 the 0 control represents 100% MTT reduction.

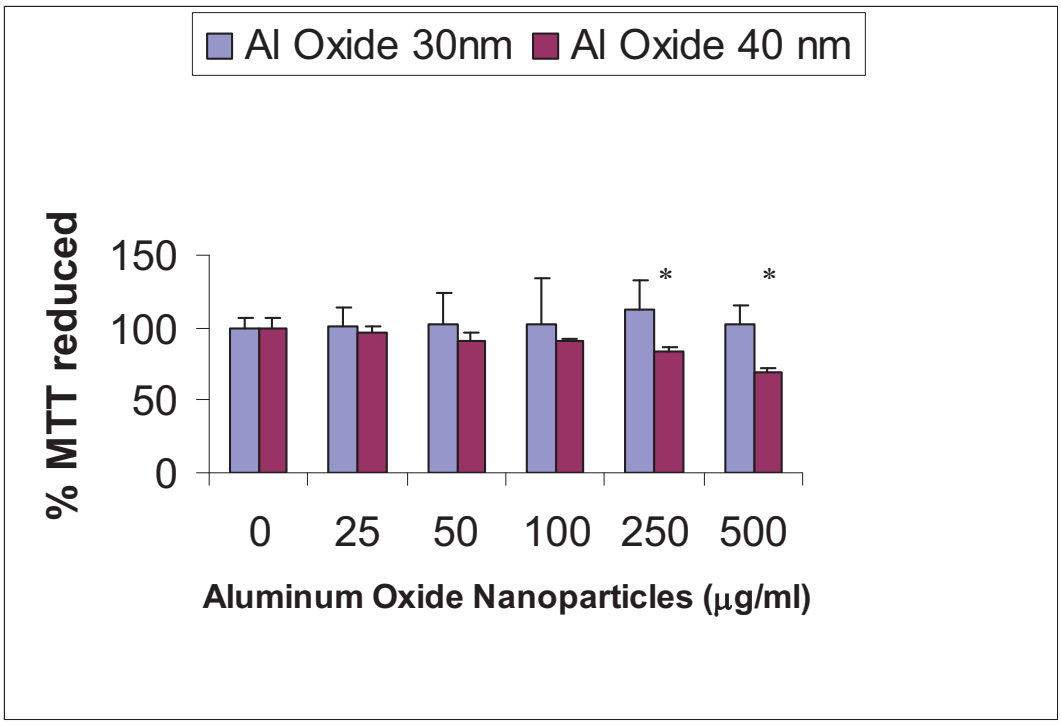
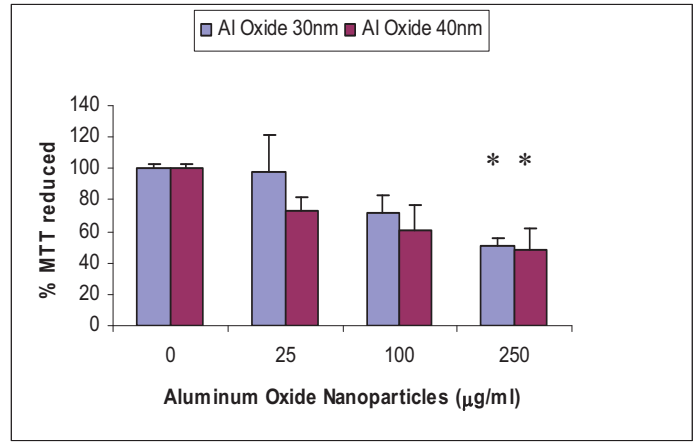


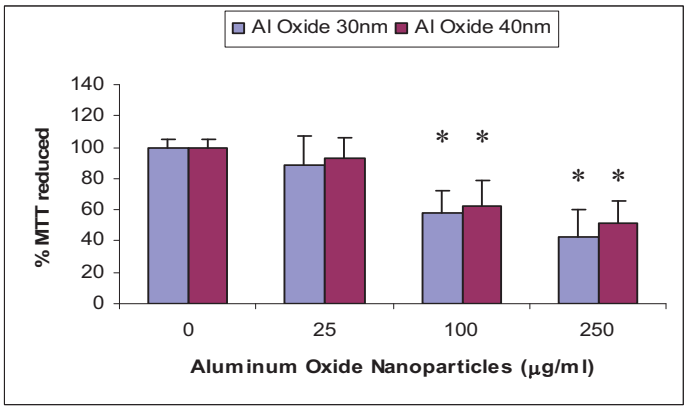
Figure 4-1: Al_2O_3-NP Effect on Macrophage Viability

Percent MTT reduced by Alveolar Macrophages after 24 h of exposure to aluminum oxide nanoparticles. (* asterisk indicates doses that are significantly different than the zero control**,** *p value* <.05)

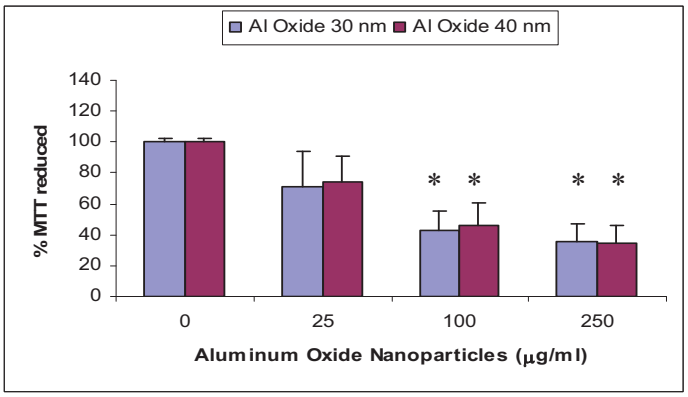
Figure 4-1 shows combined results from three separate experiments in which alveolar macrophages were exposed to aluminum oxide nanoparticle for 24 hours at doses ranging from 0 to 500 μg aluminum /ml media. These data confirms that aluminum oxide particles do not have a large impact on the viability of these cells even at concentrations as high as 500 μg/ml for exposures up to 24 hours. Aluminum oxide 40 nm at doses between 250 and 500 μg/ml were the only data points that indicate a statistical significant difference between the cells not exposed to aluminum particles (0 mg/ml or control) with a *p* value < .05. (* asterisk indicates doses that are significantly different than the zero control)



A



B



C

Figure 4-2: Al_2O_3-NP Delayed Effects on Macrophage Viability

Percent MTT reduced by Alveolar Macrophages after A) 48 h, B) 96 h, and C) 144 h of exposure to aluminum oxide nanoparticles. (* asterisk indicates doses that are significantly different than the zero control, *p value* <.05)

The amount of delayed toxicity of these aluminum oxide particles is shown in Figure 4-2. MTT reductions after 48 hrs, in both Al_2O_3 30 and 40 nm, were significant at a dose of 250 μg/ml, decreasing the amount of MTT reduction to approximately 50%. Al_2O_3 40 nm at 100 μg/ml was also significant with a decrease to 60%. MTT reduction after 96 h in both Al_2O_3 30 and 40 nm were significant at a dose of 250 μg/ml decreasing reduction to approximately 52% for 40 nm and 42% for 30 nm. Al_2O_3 40nm at 100 μg/ml was also significantly reduced to approximately 60%. MTT reduction after 144 h in both Al_2O_3 (30 and 40 nm) were significant at 250 μg/ml with a decrease to approximately 35% for both. Al_2O_3 30 nm and 40 nm at 100 μg/ml were also significant with a decrease to 43% and 45% respectively. The Al_2O_3 doses for both 30 and 40 nm were not found to be significant for the 25 μg/ml dose at any time point (*p value* > .05).

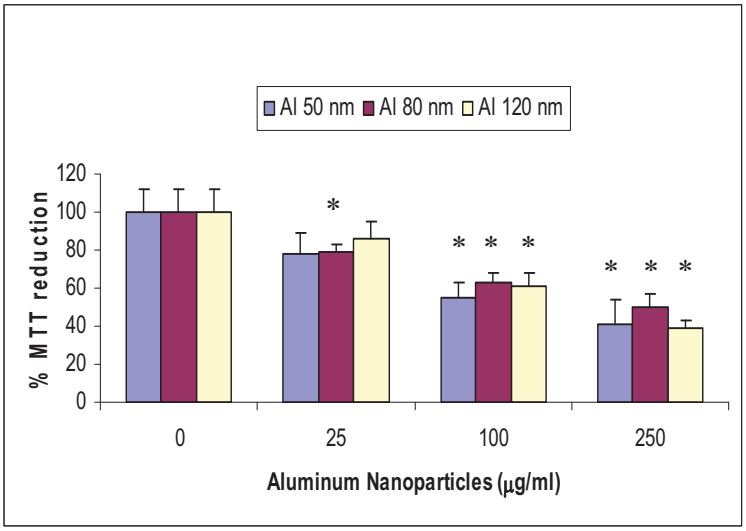


Figure 4-3: Al-NP Effect on Macrophage Viability

Percent MTT reduced by Alveolar Macrophages after 24 h of exposure to aluminum nanoparticles. (* asterisk indicates doses that are significantly different than the zero control**,** *p value* <.05)

Figure 4-3 displays percent of MTT reduction after 24 hours of exposure to aluminum nanoparticles (50, 80, 120 nm). These results indicate that aluminum nanoparticles have a more drastic effect on cell viability. Al 50 and 120 nm created a significant reduction in MTT production at 100 and 250 μg/ml, reducing MTT to 54 and 40% respectively for Al 50 nm, and 61 and 39% respectively for Al 120 nm. Al 80 nm created a significant reduction of MTT at all three dosing points. At 25 μg/ml MTT was reduced to 79%, at 100 μg/ml to 63%, and at 250 μg/ml to 49%. (* asterisk indicates doses that are significantly different than the zero control, p value$< .05$)

4.3 Alveolar Macrophage Phagocytosis Data

Phagocytic functions of these alveolar macrophages were measured by the uptake of 2 μm latex beads, observed via the use of an Olympus IX71 inverted fluorescent microscope with CytoViva as a light source (Figure 4-4).

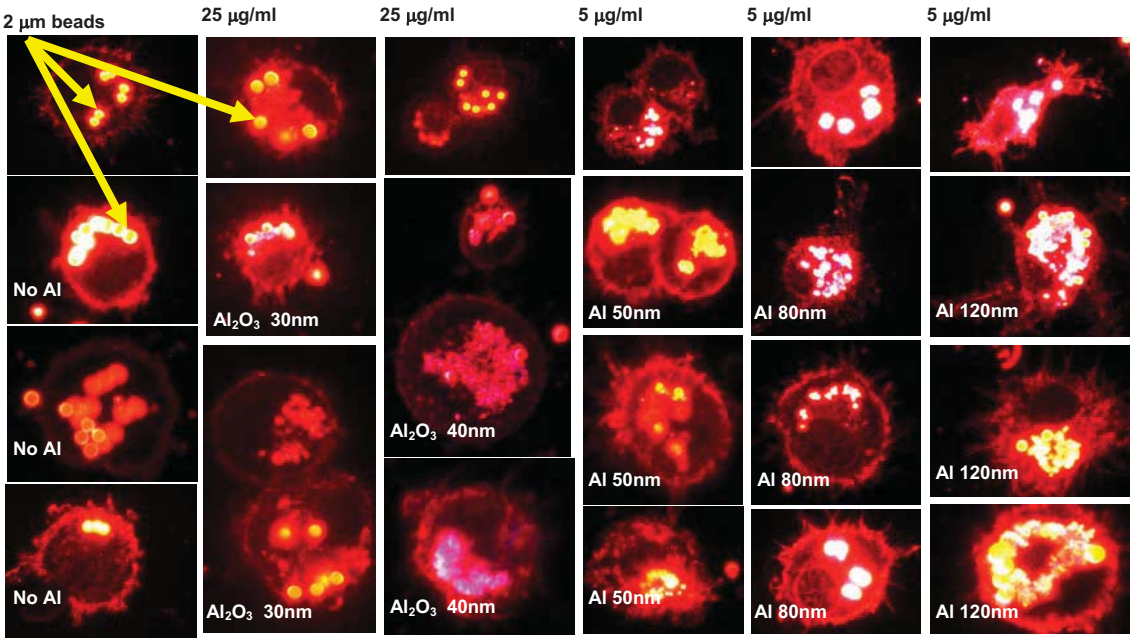
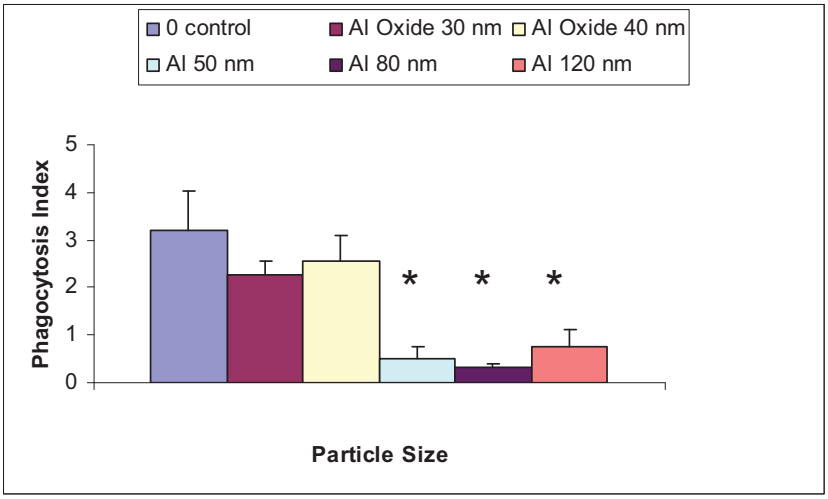


Figure 4-4: Alveolar Macrophage Images

Various images taken during phagocytosis assays with the Olympus IX71 inverted fluorescent microscope and CytoViva. Cells were exposed to Al_2O_3-NP and Al-NPs at 5 or 25 µg/ml for 24 h. 2 µm fluorescent latex beads were given to the cells after exposure. The beads appear as bright globular areas in the cells and were dosed at a 10:1 ratio (10 beads for every cell) for 6 h. Macrophages and beads phagocytosed by macrophages were counted to obtain a Phagocytosis Index (P.I). P.I. = (% macrophages that take in beads) X (average number of beads taken in by a positive macrophage)

A.



B.

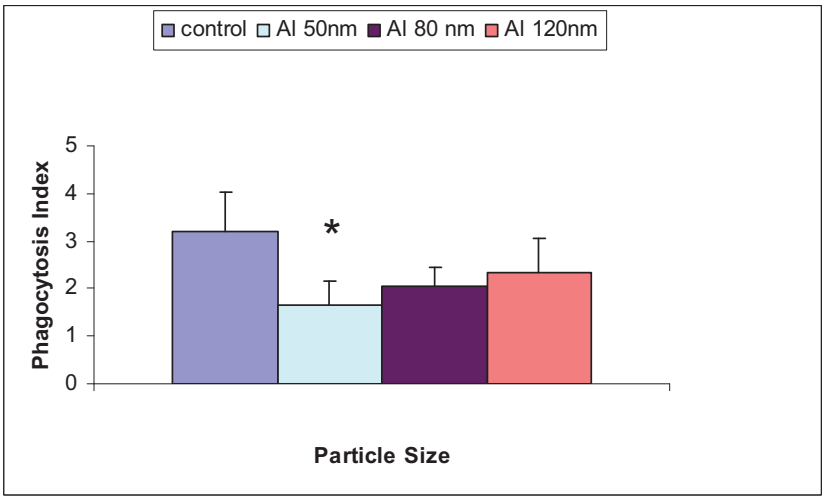


Figure 4-5: Al_2O_3-NP and Al-NP Effect on Phagocytosis Ability. A) Phagocytosis Index of Alveolar Macrophages exposed to various Al nanoparticles at 25 mg/ml for 24 hours B) Phagocytosis Index of Alveolar Macrophages exposed to various Al nanoparticles at 5 mg/ml for 24 hours (* asterisk indicates doses that are significantly different than the zero control, p value < .05)

The phagocytosis index utilized in these experiments was developed by Paine *et. al.* in 2004. Figure 4-5 shows combined results from separate experiments where alveolar macrophages were exposed to Al_2O_3-NP and Al-NP for 24 hours at a dose of 0, 5 or 25 μg aluminum /ml media. Al_2O_3-NP 30 and 40 nm showed a slight, but not significant, decrease in phagocytosis ability (p value > .05) when compared to cells not dosed with nanoparticles (0 controls). Al 50, 80, and 120 nm all showed a significant reduction in phagocytosis when compared to the control (p value < .05). Cells exposed to 5 μg/ml of Al 50, 80, and 120 nm again had a slightly reduced phagocytosis index, but only Al 50 nm was significant (p value < .05). (* asterisk indicates doses that are significantly different than the zero control, p value < .05)

4.4 Alveolar Macrophage Immune Response Data

There was no significant production of Nitric Oxide or Cytokines (TNF-alpha, MIP-2) after exposure to Al-NPs. In fact, there was statistically no difference in the amount produced at each particle size or dosing concentration up to 100 μg/ml, compared to control cells. The lipopolysaccahride (LPS) positive control illustrates that these cells were physiologically able to produce these products, but the lack of production after exposure to these nanoparticles suggests that the nanoparticles did not stimulate an immune response. This lack of an immune response suggests that inflammatory damage due to inhalation exposure of these particles is also unlikely.

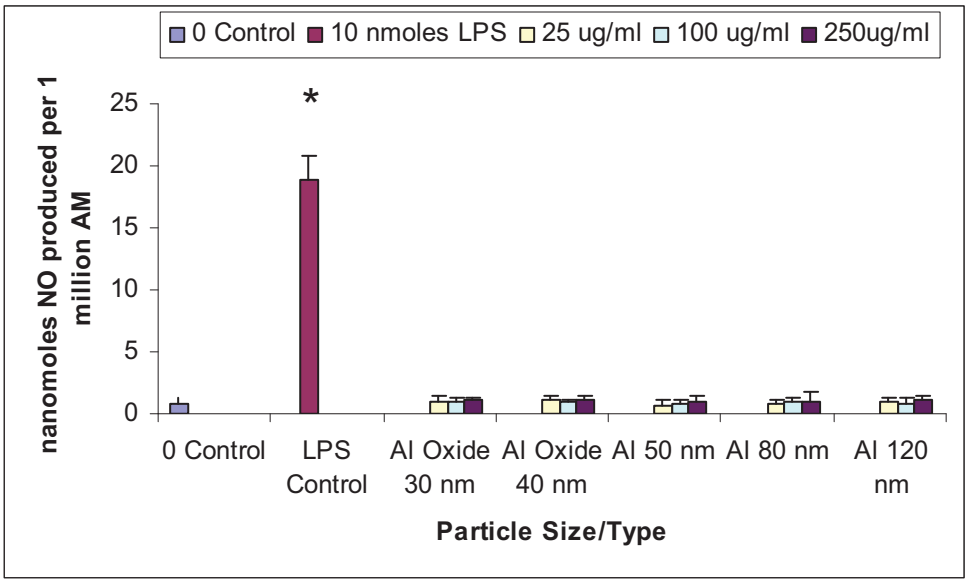
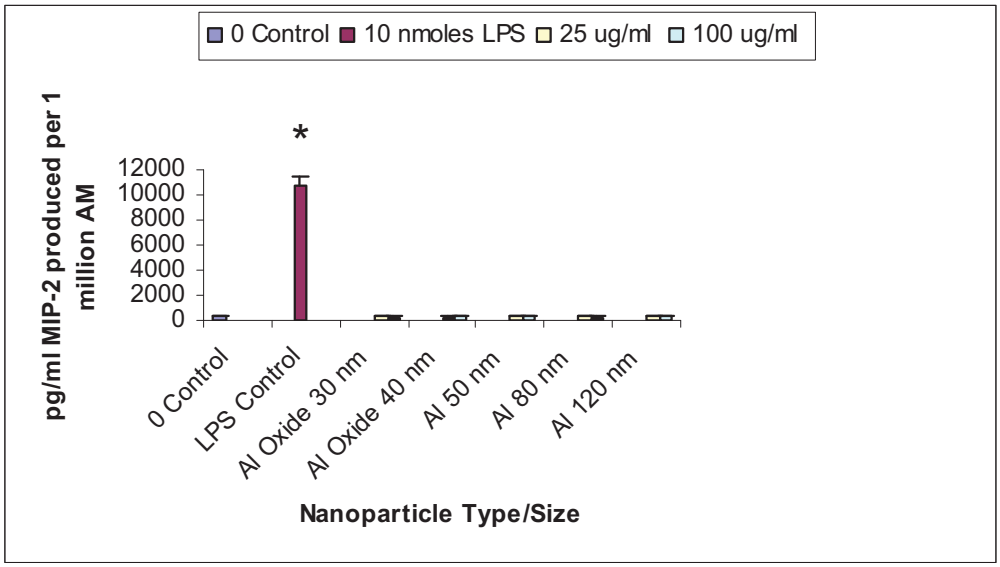


Figure 4-6: Nitric Oxide Produced by Alveolar Macrophages 24 h post Exposure to Al Nanoparticles. Lipopolysacharride (LPS) was used as a positive control**.** NO levels were determined via a Greiss reagent assay kit and performed as directed by the manufacturer (Promega). (* asterisk indicates doses that are significantly different than the zero control, p value < .05)

A.



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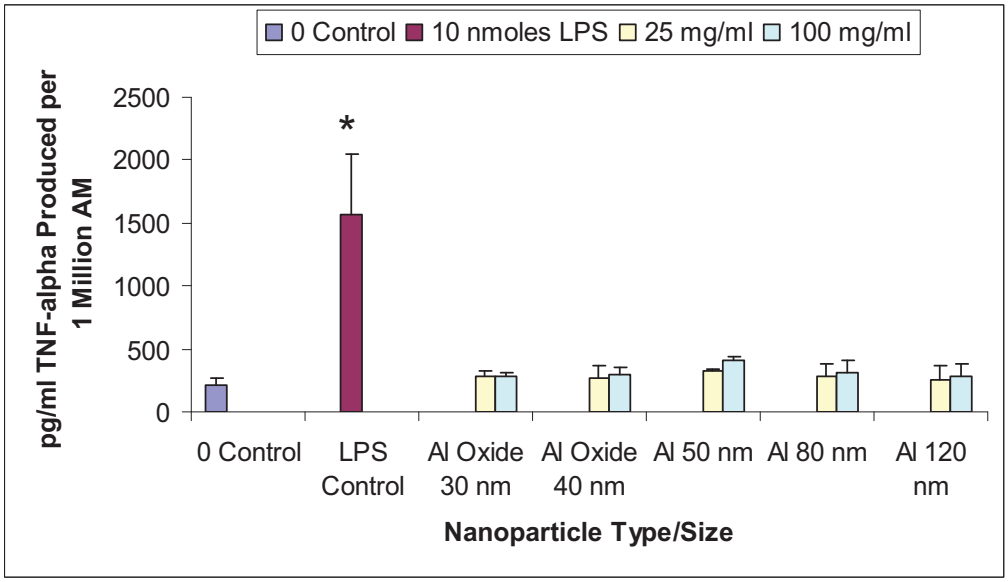


Figure 4-7: Cytokine Produced by Alveolar Macrophages 24 h Post Exposure to Al Nanoparticles. A) MIP-2 produces pg/ml, B) TNF-alpha produced pg/ml**.** Lipopolysacharride (LPS) was used as a positive control. Cytokine analysis was completed with an ELISA Kit and performed as directed by the manufacturer (Biosource). (* asterisk indicates doses that are significantly different than the zero control, p value < .05)

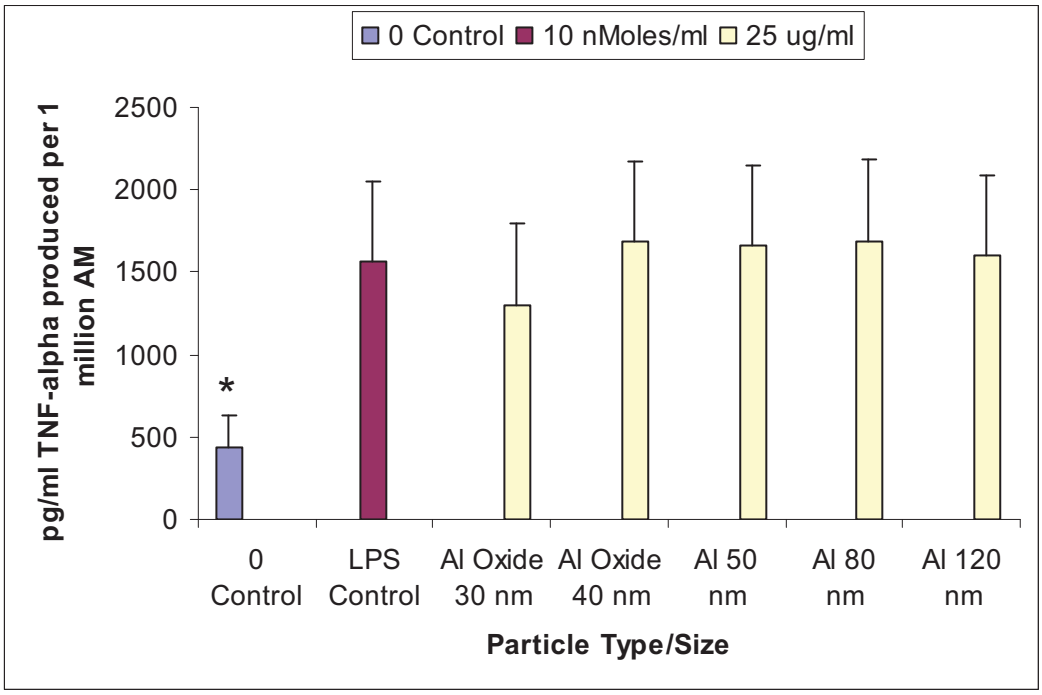


Figure 4-8: TNF-alpha Production. Alveolar Macrophages dosed with Al nanoparticles for 24 h then with Lipopolysacharride (LPS) for 24 h. TNF-alpha analysis was completed with an ELISA Kit and performed as directed by the manufacturer (Biosource). (* asterisk indicates doses that are significantly different than the LPS Control, p value < .05)

To evaluate if the particles are inhibiting the production of TNF-alpha, an additional experiment was completed with cells dosed with Al nanoparticle for 24 hrs, then dosed with the positive control agent, LPS, for 24 hrs. As seen in Figure 4-8, the cells still produced TNF-alpha. This indicates there was no particle interference with ability of the cells to produce this product and that these particles simply didn't stimulate an *in vitro* immune response that produced TNF-alpha. Additional experiments to determine if NO or MIP-2 were inhibited were not completed in this research.

4.5 Alveolar Macrophage Imaging

Three different imaging techniques were used in this research. The first was basic light microscopy with an Olympus CK2-001T microscope. Selected images from this microscope can be seen in Appendix G. This was useful in comparing viable cell numbers with MTT data in early experiments. The second method used was again light microscopy with an Olympus IX71 inverted fluorescent microscope with Cytoviva as a light source. This technique, detailed in Chapter 3, was useful in determining phagocytosis information, nanoparticle uptake, and possibly nanoparticle characterization for future research. Selected images are found in Figure 4-4. The third technique was Scanning Electron Microscopy (SEM). Selected images are found in Appendix G. It was hoped to identify nanoparticle on the outside of the cell, and compare that with TEM images of particles inside the cell, but due to limited available time with Transmission Electron Microscopy (TEM) and further protocol adjustments with SEM this was not possible. The protocol used for SEM found in Chapter 3 is a good start for future research in this area.

V. Conclusions and Future Research Considerations

5.1 Overview

This chapter summarizes the final results of this research, suggests additional research to better understand effects of nanoparticles on cells, discusses methodology improvements, and discusses DoD's interests concerning health and nanotechnology.

This research compared the *in vitro* response of Rat Alveolar Macrophages to various sizes of aluminum nanoparticles to determine toxic effects on cell viability and functionality. Cell viability was assessed via the MTT assay, measuring the metabolic activity of mitochondria. The functionality of these cells was assessed with both phagocytosis and cytokine production assays. In addition to these assays, advances in imaging these cells were made during this research.

5.2 Conclusions

5.2.1 Viability and Respiration

Al_2O_3-NPs did not display significant toxicity after 24 hours of exposure to the AM cells, but after extended times, 96 and 144 hours, at higher doses, 100 and 250 μg/ml, a statistically significant reduction was seen in the metabolic activity of the cells. Al-NPs were slightly more toxic than Al_2O_3-NPs after 24 hours at higher doses, 100 and 250 μg/ml.

The slight decrease of MTT reduction in cells exposed to 40 nm Al_2O_3 and the unaffected reduction of MTT by cells exposed to high doses of 30 nm Al_2O_3 indicate only slight to no effect on AM cell viability after 24 hours of exposure. A delayed effect on viability was shown to occur between 96 and 144 hours, at high exposure levels for.

Al_2O_3-NP. Delayed toxicity for Al-NP was not investigated since significant toxicity was seen after 24 h.

The percent MTT reduction in the Al-NP and Al_2O_3-NP did not seem to be size or surface area dependent, see surface area figure for each nanoparticle size in appendix L. The major difference in viability seemed to be the result of the chemical composition of the nanoparticles or possibly other characteristics not investigated. The same seemed to be true for the phagocytosis ability of these macrophages after exposure to Al_2O_3-NP and Al-NP for 24 h. The AL-NPs seem to restrict the phagocytic function of the cell more than Al_2O_3-NP, and again, there were no statistical differences between different Al-NP sizes.

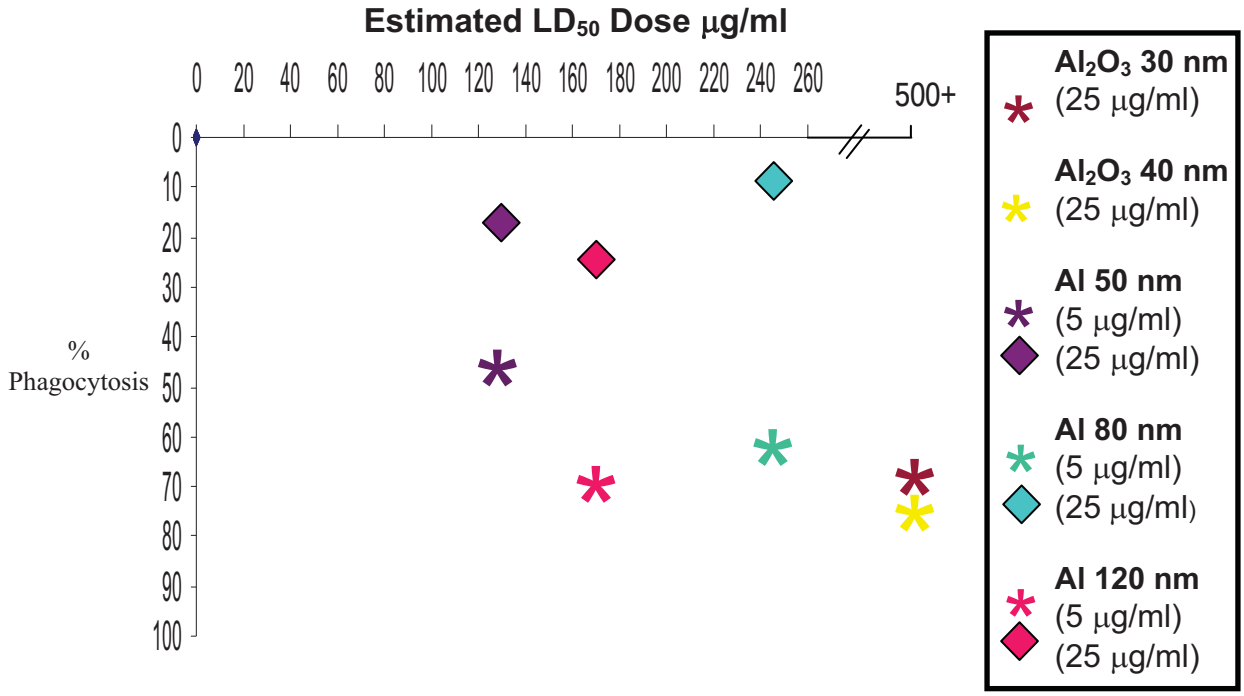


Figure 5-1 LD_{50} Dose Estimates and Low Exposure Phagocytosis Reduction

X-axis shows LD_{50} dose estimates for these aluminum nanoparticles on AM. Y-axis shows a significant reduction in the % phagacytosis ability of AMs exposed to Al nanoparticles compared to AMs not exposed.

5.2.2 Phagocytosis

Phagocytosis assays were done to examine the possibility that these particles will have an effect on the function of phagocytosis AM. As shown in Figure 5-1 the estimated LD_{50} dose for each nanoparticle was relatively high. 50 nm Al-NPs were the most toxic with an estimated LD_{50} dose of 130μg/ml and Al Oxide 30 and 40 nm were both well above 500 μg/ml. Since AM cell viability was not significantly affected at lower exposure levels, these additional assays were run to measure the cell's functional abilities. When cells were dosed at lower, non toxic levels (25 μg/ml), Al-NPs all caused a statistically significant reduction in phagocytosis. Even at 5 μg/ml, 50 nm Al-NP caused a 48% reduction in phagocytosis that was statistically significant compared to the control**.** The inability of the cells to function properly could contribute to the onset of infection since these impaired macrophages will not be able to quickly clear bacteria that make it to the alveoli (Lundborg, *et al.*, 2001). In addition, synergistic effects might occur between materials and particles that were in the air with Al-NPs. Previously non-toxic materials or particles, usually consumed by macrophages, could contribute to fibrosis or other diseases in the lungs if they are not removed.

5.2.3 Immune Response

The immune response of alveolar macrophages to aluminum nanoparticles was measured by determining the degree of nitric oxide and cytokine (TNF-alpha, MIP-2) production by the cells. Increased immune response would indicate potential for inflammatory damage to tissue. Lipopolysaccharide was used as a positive control and

demonstrated these cells do produce these inflammatory products at expected levels. Exposure to each size of aluminum nanoparticles produced no statistical difference between the amount of cytokine or nitric oxide produced from cells exposed compared to cells not exposed.

5.2.4 Imaging

Various techniques for imaging cells and their interactions with nanoparticles were explored. These included basic light microscopy, fluorescent light microscopy with CytoViva, and a procedure for Scanning Electron Microscopy (SEM), see Appendix G for various images. Each of these techniques was useful in directing the focus of this research. Basic light microscopy was critical in completing cell counts, observing cell confluency, and determining initial nanoparticle doses that had an effect on cell viability prior to and in conjuction with the MTT assays. The fluorescent light microscope proved to be very useful in determining the phagocytosis ability of the AM cells and observing the extent to which these nanoparticles agglomerated. The SEM imaging, not as successful, was used to gain more information on cell morphology and particle location on the cell membrane. Image quality and instrument availability was problematic, but a few images did show likely nanoparticle agglomerations on the outside of cells. Additional technique modifications with SEM along with TEM imaging will be needed in order to determine nanoparticle locations in respect to the AM cells and overall changes in AM cell morphology.

5.3 Recommended Additional Research

Nanotechnology interactions with environmental and human health still need to be addressed. This research has uncovered additional concerns with respect to *in vitro* studies that are beyond the scope of this thesis. This section discusses recommendations for additional studies, ranging from particle uptake and characterization to regulation and control of these nanoparticles during mass production or release.

5.3.1 Particle Uptake

One question that is a concern and leads to numerous others is the issue of uptake of nanoparticles in exposed cells. Are the particles agglomerated on the cell membrane surface or are they crossing the membrane? If the later, how are they getting in: phagocytosis, endocytosis, physical trauma, etc.; then where are they going: cytoskeletol elements, nucleus, ribosomes, etc.; and finally, are they interfering with physiological processes such as: protein synthesis, DNA replication, cell locomotion, etc? First, better techniques to determine concentration of particles in the cells after they have been exposed at various exposure concentrations and times are needed. Second, precise particle locations inside the cells need to be evaluated to determine any functional changes, beyond death, that these nanoparticles might have on the cell.

This is a difficult task that could possibly be accomplished with common technologies. SEM and TEM have been used to determine where particles are in relation to the cell and its organelles. Techniques that use atomic absorption and inductive coupled plasma are now being explored to determine mass concentration of metallic nanoparticles in cells. Fluorescent studies with nano-sized latex beads could also be used

to model uptake. Appendix I illustrates two proposed methods for Nanoparticle uptake that utilize atomic absorption and fluorescent latex beads.

5.3.2 Mechanism of Cell Death

An additional question is the cause of death after exposure. Fink *et al.* in 2005 explains the differences between apoptosis, a type of self programmed cell dismantling that avoids inflammatory responses, and necrosis, a passive accidental death that can lead to uncontrolled inflammatory response. The method of death influences tissue damage due to inflammation. Cell responses like mitochondrial membrane potential, annexin binding, DNA fragmentation, caspase activation, and cytochrome C release can be measured in order to determine what type of cell death is predominant.

5.3.3 Characterization and Exposure

A limitation of nanoparticle toxicology has been the lack of physical characterization of the particles that the cells are exposed to. Recently Oberdorster *et al.* (2005) addressed this issue and determined certain key characteristics; size distribution, shape, composition, particle physicochemical structure, agglomeration state, method of production, storage of material, and concentration, are essential characteristics that need to be evaluated. Slight change in any of these factors may cause nanoparticles to be more or less toxic. It is still widely believed by many that, due to small particle size, an increased surface area interaction is one of the most important factors. If a manufacturing process creates particles of the same size, but a slightly different surface chemistry, this could be crucial to toxicity. Results from this research showed Al-NPs to be more toxic than Al_2O_3-NPs.

Another big factor is the method of exposure of cells to particles. Do the methods and procedures used mimic *in vivo* exposure, and, if so, have any of the nanoparticle characteristics changed in the process? For example, particles dispersed in water may have different properties than particles dispersed in PBS or media. Particles in high dose concentrations may have different properties than particles at low concentrations. Particles agitated prior to exposure may have different properties than particles not agitated. This can become an important issue in how often particles are characterized, how reliable are the results, and how much time and money should be spent on this?

A synergistic effect may occur between materials and particles that are in the air with Al-NP. Since nanoparticles may impair alveolar macrophage function, as was seen by Al-NPs, effects of other materials commonly aerosolized with nanoparticles during production, handling, and use should be investigated. Materials or particles, usually consumed by macrophages and previously thought harmless, may contribute to fibrosis, infection, or other diseases in the lungs.

5.3.4 Modeling

Once enough data is collected, an attempt to model cellular effects should be made. This may be useful in finding trends associated with the physically characteristics of the particles (size, shape, chemistry, etc.), the type of cells effected, or various other biological interactions. These trends may be useful in hypothesizing the toxic effects of future nanoparticles even before they are used. First, *in vitro* data from numerous cell lines including skin, lung, kidney, liver, and digestive tissues should be collected. Second, data should include both viability and functional effects of the nanoparticles on the cells. Third, the nanoparticles used should be highly characterized according to all elements explained by Oberdorster *et al.* (2005). Finally, the hope is that good modeling

should lead to further studies and offer information on *in vivo* exposure levels. These in vivo exposure levels could eventually lead to identifying exposure limitations that should be made in a work environment.

5.3.5 Future DoD Applications and Concerns

A big financial push has been made toward nanotechnology. As illustrated in the 2005 report on Defense Nanotechnology Research and Development Programs, over 230 million dollars (annually) has been allotted toward R&D for nanotechnology in DoD. The fact is these materials, if not already, will soon be in our work place. A better evaluation of exposure limitations and environmental measurement techniques need to be addressed. A collaborative effort between DoD, private industry and NIOSH on this matter should be investigated. DoD as well as numerous other companies have an interest in production of nanomaterials other that titanium oxide and carbon black, but the current NIOSH strategic plan seems to have a limited focus on these two particle types, see Appendix H (NIOSH strategic plan, 2005).

A proactive effort should also be made by DoD on how these materials are to be regulated (see Appendix J). In a 2003 Case study of Single Walled Carbon Nanotubes, completed by the Woodrow Wilson International Center for Scholars, numerous loop holes where found in how well the Toxic Substance Control Act (TSCA) monitored the production of these new materials (Nanotechnology and Regulation Case study, 2003). Research should be conducted to determine the best nanoparticle use policy that protects the worker and the environment while limiting the potential of nanotechnology as little as possible.

Appendix A: Trypsinizing Cells

In order to maintain adequate conditions for cell growth and harvesting the cells had to be removed from their collagen matrix with trypsin.

1. Trypsin was brought to room temperature to avoid stress to the cells
2. Growth media was suctioned from each flask
3. 1 ml of .25% trypsin was injected into each flask and allowed to coat the entire surface of the flask and incubated for 1-2 minutes
4. 5 ml media was added to each flask and aspirated with a pipette over the entire surface of the flask many times to allow for the cells to be washed from their collagen matrix
5. A desired volume of media containing cells was placed into another flask, or counted to determine a concentration of cells, and seeded to a 6 or 24 well plate for an assay, or to a slide for microscopic examination.
6. All plates, flasks, and slides were allowed to incubate for 2-3 hours to allow for cells to reattach to the collagen substrate.
7. The final step of the process was to suction out all media that contained trypsin and re-suspend cells in fresh media, to allow cells to grow

Appendix B: MTT Assay

The MTT assay was used to establish alveolar macrophage viability before and after exposure to Al nanoparticles.

1. Macrophages were seeded on collagen coated 24 well plates at 5 x 10^5 AM/mL in 20% FBS Media 48 hours prior to desired assay.

2. 24 hours prior to assay, stock solution of nanoparticles were sonicated for 20 seconds, and suspend in 20% FBS to attain desired nanoparticle media concentration

3. Media was replaced with nanoparticle/media solutions and incubated at 37 degrees Celsius

    a. For a 24 hr end point, the MTT assay, as explained below, was completed the next day at the same time
    b. For the 48, 96, 144 hr end point the MTT assay was completed as explained below, but the media/nanoparticle solution was suctioned out and replaced with pure 20% FBS media after 24 hrs, and each assay was performed 48, 96 or 144 hr after initial exposure to the particles

4. In the 24 well plates, 100 µl of MTT stock was added directly into the media in each well.

5. The plate was incubated at 37° C until color developed (usually 30 minutes).

6. The solution was re-suspended and transferred into 1.5 ml tubes.

7. The tubes were centrifuge for 2 minutes at 2,000 x g.

8. The supernatant was removed and 0.5 ml of 70% isopropanol was added to each tube with pellet.

10. The pellet was vortexed well to obtain homogeneous staining.

11. 200 µl of the samples were transferred into wells of a 96 well plate.

12. Plates were read on the Spectomax 190 at 570 nm to 630 nm.

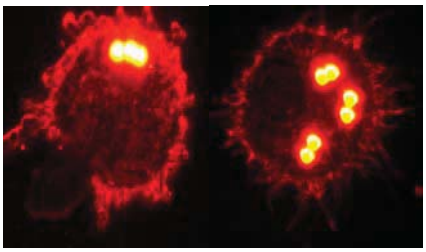
13. Percent reduction is compared to the 0 or blank control.
    (0 control represents 100% MTT reduction)

Appendix C: Phagocytosis Assay

AFRL SOP Draft

AL/OET **SOP NO: (Draft)**
Standard Operating Procedure (Draft)
Author: 1st Lt. Andrew Wagner
Date: 26 September 2005

PHAGOCYTOSIS ASSAY



PURPOSE/PRINCIPLE: This standard operating procedure describes the in vitro method of determining what effects different nanomaterials will have on the phagocytic function of alveolar macrophages.

KEY WORDS: Phagocytosis, alveolar macrophages, nanotoxicity.

1. SAFETY AND OPERATING PRECAUTIONS:

1.1 Wear appropriate gloves, eye goggles and lab coat when handling hazardous materials.

1.2 Properly dispose of unused/spent toxic materials (nanoparticles).

2. EQUIPMENT/MATERIALS:

2.1 F12K medium supplemented with 20 % fetal bovine serum (FBS), 1 % penicillin and streptomycin.

2.2 2 chambered microscope slides, and clean glass microscope cover slips

2.3 2 μm latex beads (Sigma Aldrich Product #L3030, (www.sigmaaldrich.com/catalog/search/SearchResultsPage/Expand)

2.4 Nanomaterials of interest

2.5 Olympus IX71 Inverted Fluorescent Microscope with CytoViva 150 attachment and 12 bit QICAM from IMAGINE (Lab 160)

3. SPECIMEN/SAMPLE: 250,000 Alveolar Macrophages in 1ml media

4. REAGENTS: N/A

5. PROCEDURE:

5. 1 Seed macrophages on collagen coated 2 chambered slides at a low density approximately 2.5 x 10^5 AM/ml in media containing 20% FBS for 2-4 hours prior to step 5.2

5.2 24 hour (or other desired time period) prior to microscopic evaluation suspend desired nanomaterials in cultured media with 20% FBS to attain desired material concentration in cultured media.

5.3 Expose cells to the different concentrations of nanomaterials in cultured media. After a desired time period of exposure cell concentrations are determined by washing selected wells with Media. The cells are washed off their collagen substrate and counted via the cell counting method explained previously by Dr. N. DelRaso (30 Jan 1995).

5.4 Once cell concentrations are determined a bead concentration ratio of 10 beads to 1 cell (2 μm Beads: number of cells) in 20% FBS media is used to replace the nanomaterial/media solution in the slide chambers that were not counted

5.5 Cells expose to beads in cultured media are then incubated for 6 h at 37^{o}C.

5.6 After 6 h of incubation, cells are washed twice with media at room temperature. The chambers on the slides are removed as described in the manufacturer's instruction and a cover slip is placed on top of the sample. The edges of the cover slip are adhered to the slide and are sealed with fingernail polish to avoid movement.

5.7 Cells should first be counted with the CytoViva light on while fluorescence light is off.

5.8 Beads inside cells can then be counted with fluorescence and CytoViva on. Open and close the fluorescent shutter as needed and focus with the fine adjustment to view and count all beads in a cell

5.9 Start the cell count in the middle of the slide and count every cell in a field of view and the number of beads within each cell. Once all cells are counted move the field of view up the slide vertically and once again count each cell. If the edge of the slide appears before 100 cells are counted move the field of view horizontally and then proceed down vertically counting.

6. DATA ANALYSIS/RECORDS:

6.1 A Phagocytosis Index (PI) is determined after 100 cells are counted.
(PI = percent macrophages that took beads in multiplied by the average number of beads taken in by a positive macrophage)

6.2 The average number of beads taken in by a positive macrophage and the average number of positive macrophages are additional data points that can be used.

7. QUALITY CONTROL:

7.1 Repeat Experiment at least three times for a total cell count of 300 cells for each nanoparticle exposure and dose.

8. LIMITATIONS OF PROCEDURE: N/A

9. PROCEDURE NOTES: N/A

10. REFERENCES:

Renwick, L.C., K. Donaldson, and A. Clouter (2001). Impairment of Alveolar macrophage Phagocytosis by Ultrafine Particles, Toxicology and Applied Pharmacology **172,** 119–127

Paine, R., Morris, S.B., Hong, J., Wilcoxen, S.E., Phare, S.M., Moore, B.B., Coffey, M.J., Toews, G.B., 2001, Impaired functional activity of alveolar macrophages from GM-CSF-deficient mice. *American Journal of Physiology Lung Cell Molecular Physiology* 281: 1210-18

11. ADDENDA/ATTACHMENTS: N/A

Appendix D:Nitric Oxide Assay

This nitric oxide assay was used to characterize effects of Al nanoparticles on an Alveolar Macrophages inflammatory response.

1. Macrophages were seeded on collagen coated 6 well plates with 5 x 10^5 AM/mL in 20% FBS media for 48 hours prior to desired assay time

2. 24 hours prior to assay time the stock solution of nanoparticles were sonicate for 20 seconds, and suspended in 20% FBS to attain desired nanoparticle media concentrations.

3. Media was replaced with nanoparticle/media solutions. The wells were incubated for 24 hours

4. After 24 hour exposure to the nanoparticles cell concentrations in the slide wells were determined by washing selected wells with Media. The cells were washed off their collagen substrate and counted via the cell counting method explained in 3.5

5. Cells were scraped off the bottom of each well.

6. Cells and supernatant were sonicated for 5 seconds

7. Cells and supernatant were centrifuged for 8 minutes at 1200g

8. Nitric Oxide assay performed as directed by manufacturer (Promega)

9. A nitrite standard curve was created:

    A. A 100µM nitrite solution was prepared by diluting the 0.1M Nitrite Standard 1:1,000 in the matrix or buffer used for the experimental samples.

    B. Three columns (24 wells) in a 96-well plate were designated for the Nitrite Standard reference curve (Figure A-1). 50µl of the appropriate matrix or buffer was dispensed into the wells in rows B.H.

    C. 100µl of the 100µM nitrite solution was added to the remaining 3 wells in row A.

    D. Serial two fold dilutions (50µl/well) in triplicate, were performed down the plate to generate the Nitrite Standard reference curve (100, 50, 25, 12.5, 6.25, 3.13 and 1.56µM), discarding 50µl from the 1.56µM set of wells. No nitrite solution was added to the last set of wells (0µM).

10. Nitrite Measurement (Griess Reaction):

A. Sulfanilamide Solution and NED Solution was allowed to equilibrate to room temperature (15-30 minutes).

B. 50µl of each experimental sample was added to wells in duplicate or triplicate.

C. 50µl of the Sulfanilamide Solution was added to all experimental samples and wells containing the dilution series for the Nitrite Standard reference curve.

D. Samples were incubate 5-10 minutes at room temperature, protected from light.

E. 50µl of the NED Solution was added to all wells.

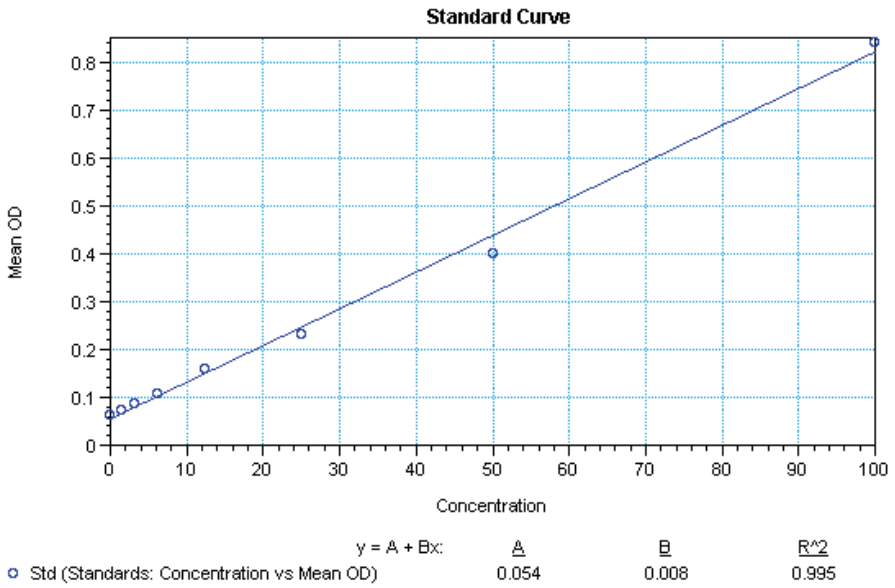
F. Samples were incubate 5-10 minutes at room temperature, protected from light.

G. Absorbance was measure on the Spectromax 190 within 30 minutes with a Filter between 520-550nm.

11. Determination of Nitrite Concentrations in Experimental Samples:

A. A Nitrite Standard reference curve was generated by plotting the average absorbance value of each concentration of the Nitrite Standard as a function of "Y" with nitrite concentration as a function of "X".

B. The average absorbance value of each experimental sample was determined. Sample concentrations were determined by comparison to the Nitrite Standard reference curve.



Appendix D Figure A: Nitrate Standard reference curve used to calculate unknown sample concentrations according to absorbance

Appendix E: TNF-alpha Assay

This TNF-alpha assay was used to characterize effects of Al nanoparticles on

Alveolar Macrophages inflammatory response

1. Macrophages were seeded on collagen coated 6 well plates with 5 x 10^5 AM/ml in 20% FBS media for 48 hours

2. 24 hours prior to supernatant collection the stock solution of nanoparticles were sonicate for 20 seconds, and suspended in 20% FBS to attain desired nanoparticle media concentrations.

3. Media was replaced with nanoparticle/media solutions. The wells were incubated for 24 hours

4. After 24 hour exposure to the nanoparticles cell concentrations in the 6 well plates were determined by. The cells were washed off their collagen substrate and counted via the cell counting method explained in 3.5

5. Supernatant media from each of the wells not counted were collected and placed into 1.5 ml tubes and frozen at -20 degree Celsius for future use.

6. Supernant liquids were thawed at room temperature and centrifuged at 1200 g for 5 minutes.

7. 1:4 dilutions of the supernants were used (determined by previous experiments) as samples and the TNF-alpha assays were performed as directed by the manufacturer (Biosource) with slight modifications

9. A TNF-alpha standard curve was created: (see Figure A-2)

    a. The standard provided was reconstituted to 5000 pg/ml with Standard Diluent Buffer.
    b. 0.100 ml of the reconstituted standard was added to a tube containing 0.400 ml Standard Diluent Buffer
    c. 0.250 ml of Standard Diluent Buffer was added to each of 6 tubes labeled 500, 250, 125, 62.5, 31.2 and 15.6 pg/ml Rat TNF-alpha.
    d. Serial dilutions of the standard were made.

10. The desired number of 8 well strips was inserted into the frame provided

11. 50 µL of the Incubation Buffer was added to all wells.

12. 100 µL of the Standard Diluent Buffer was added to the zero wells.

13. 100 µL of standards or controls was added to the appropriate wells, and 100 µL of sample to each well.

14. 50 µL of biotinylated anti-TNF-α (Biotin Conjugate) Solution was pipetted into each well.

15. Plates were covered and incubate for 1 hour and 30 minutes at room temperature.

16. Solutions were discarded and washed four times with wash solution.

17. 100 µL of Streptavidin-HRP working solution were added to each well.

18. Plates were covered and incubate for 45 minutes at room temperature.

19. Solutions were discarded and washed four times with wash solution.

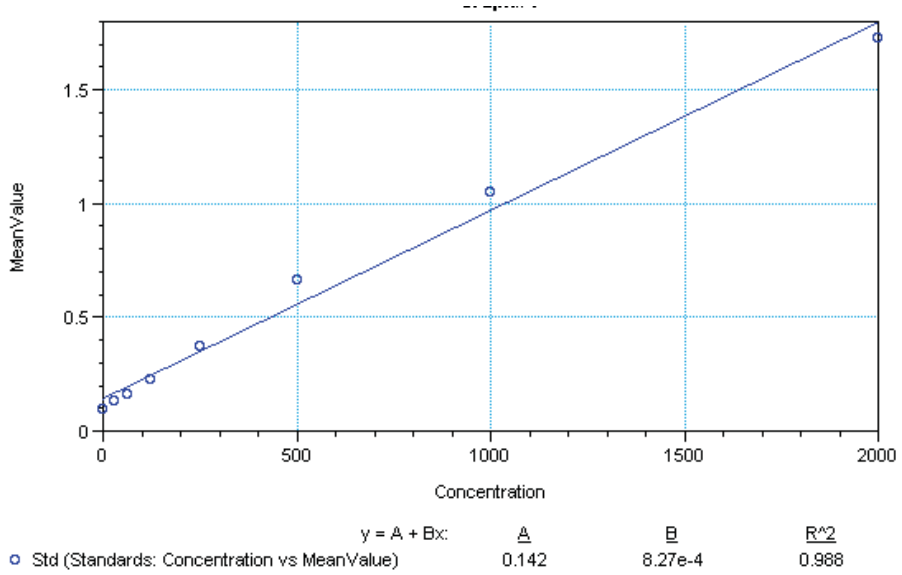
20. 100 µL of Stabilized Chromogen was added to each well.

21. The wells were incubated for about 15 minutes at room temperature and in the dark.

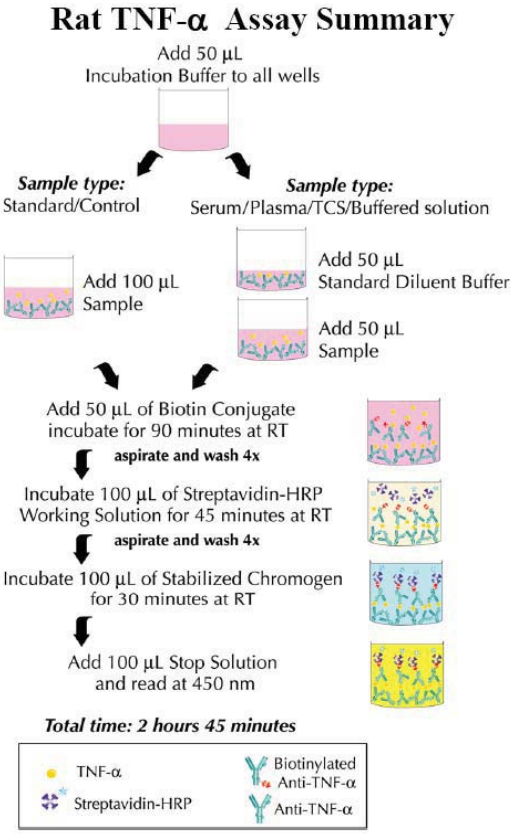
22. 100 µL of Stop Solution was added to each well. (The solution in the wells should change from blue to yellow).

23. The absorbance of each well was read at 450 nm.

24. The absorbance data was compared to the standard TNF-alpha curve and concentrations were determined



Appendix E Figure A: TNF-alpha Standard Reference Curve used to calculate unknown sample concentrations



Appendix E Figure B: Biosource Immunoassay Kit Catalog # KRC3011C, 2004, *Rat* TNF-alpha

Appendix F: MIP-2 Assay

This MIP-2 assay was used to characterize effects of Al nanoparticles on Alveolar Macrophages inflammatory response

1. Macrophages were seeded on collagen coated 6 well plates with 5 x 10^5 AM/ml in 20% FBS media for 48 hours

2. 24 hours prior to supernatant collection the stock solution of nanoparticles were sonicate for 20 seconds, and suspended in 20% FBS to attain desired nanoparticle media concentrations.

3. Media was suctioned replaced with nanoparticle/media solutions. The wells were incubated for 24 hours

4. After 24 hour exposure to the nanoparticles cell concentrations in the 6 well plates were determined. The cells were washed off their collagen substrate and counted via the cell counting method explained in 3.5

5. Supernat media from each of the wells not counted were collected and placed into 1.5 ml tubes and frozen at -20 degree Celsius for future use.

6. Supernant liquids were thawed at room temperature and centrifuged at 1200 g for 5 minutes.

7. 1-10 dilutions of the supernants were used (determined by previous experiments) as samples and the MIP-2 assays were performed as directed by the manufacturer (Biosource) with slight modifications

9. A MIP-2 Standard Curve was created: (see Figure A-3)

    a. The standard provided was reconstituted to 2000 pg/ml with Standard Diluent Buffer.
    b. 0.160 ml of the reconstituted standard was added to a tube containing 0.340 ml Standard Diluent Buffer
    c. 0.150 ml of Standard Diluent Buffer was added to 6 tubes labeled 320, 160, 80, 40, 20 and 10 pg/ml Rat MIP-2.
    d. Serial dilutions of the standard were made.

10. The desired number of 8 well strips was inserted into the frame provided

11. 50 μL of the Standard Diluent Buffer was added to the zero wells.

12. 50 μL of standards or controls were added to the appropriate wells, and 50 μL of sample to each well.

13. 50 µL of biotinylated anti-MIP-2 (Biotin Conjugate) Solution was pipetted into each well.

14. Plates were covered and incubate for 2 hours at 37 degree Celsius.

16. Solutions were discarded and washed four times with wash solution.

17. 100 µL of Streptavidin-HRP working solution was added to each well.

18. Plates were covered and incubate for 1 hour at room temperature.

19. Solutions were discarded and washed four times with wash solution.

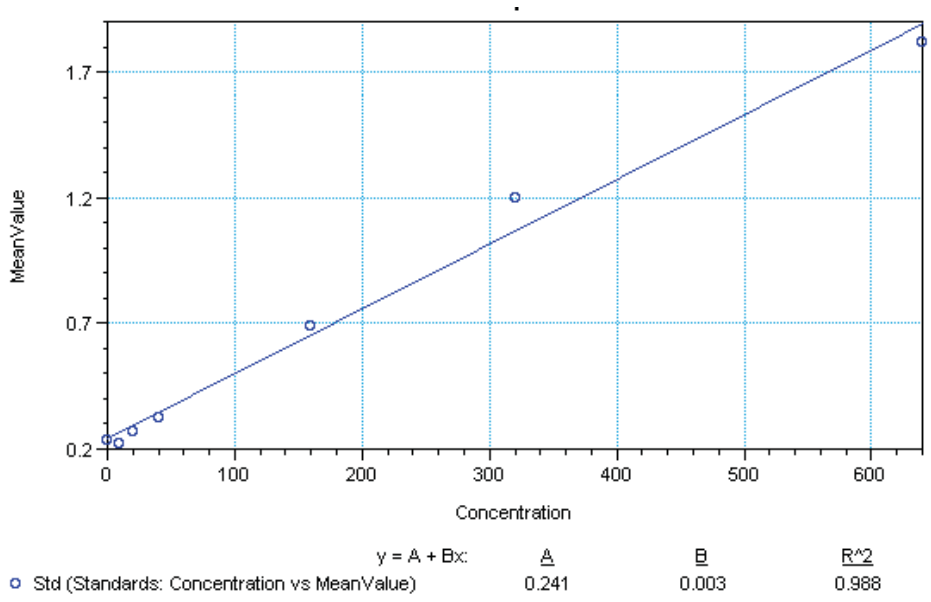
20. 100 µL of Stabilized Chromogen was added to each well.

21. The wells were incubated for about 15 minutes at room temperature and in the dark.

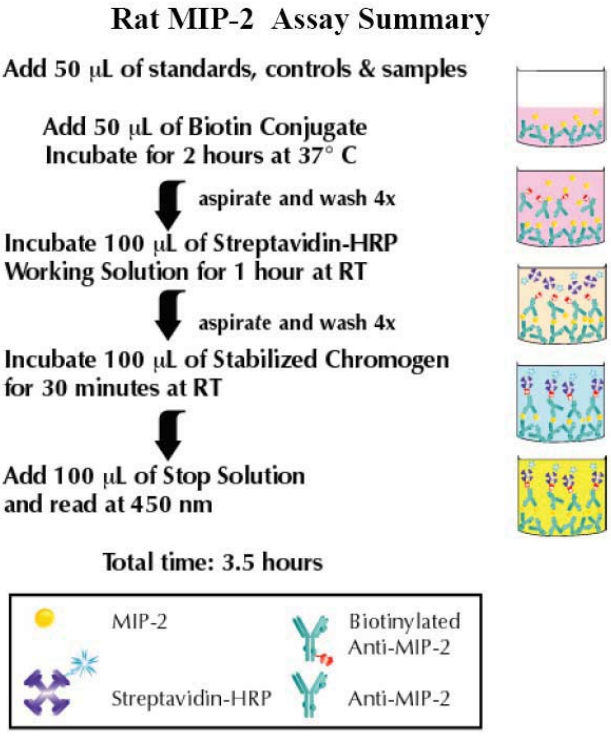
22. 100 µL of Stop Solution was added to each well. (The solution in the wells should change from blue to yellow).

23. The absorbance of each well was read at 450 nm.

24. The absorbance data was compared to the standard MIP-2 curve and concentrations were determined.

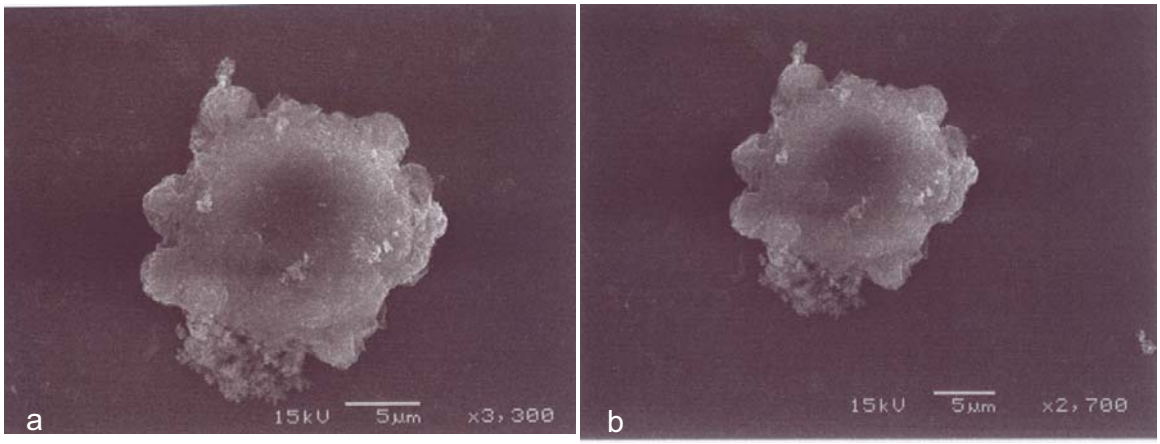


Appendix F Figure A: MIP-2 Standard Reference Curve used to calculate unknown sample concentrations

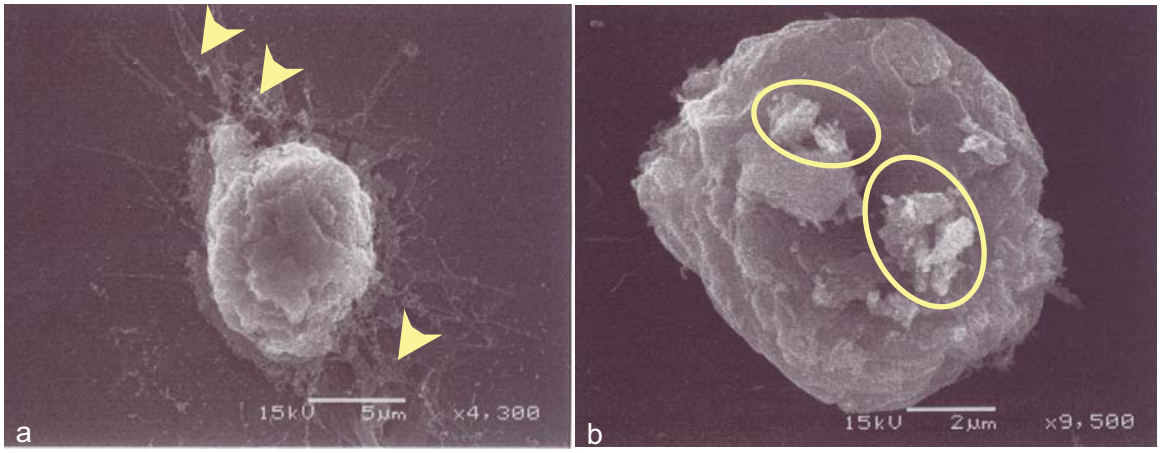


Appendix F Figure B: Biosource Immunoassay Kit Catalog #KRC1022/KRC1021, 2003, *Rat* MIP-2.

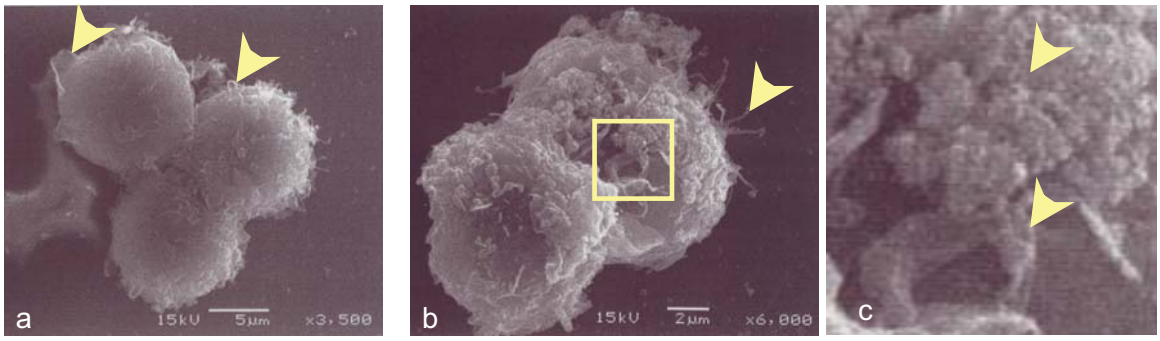
Appendix G: Selected Images



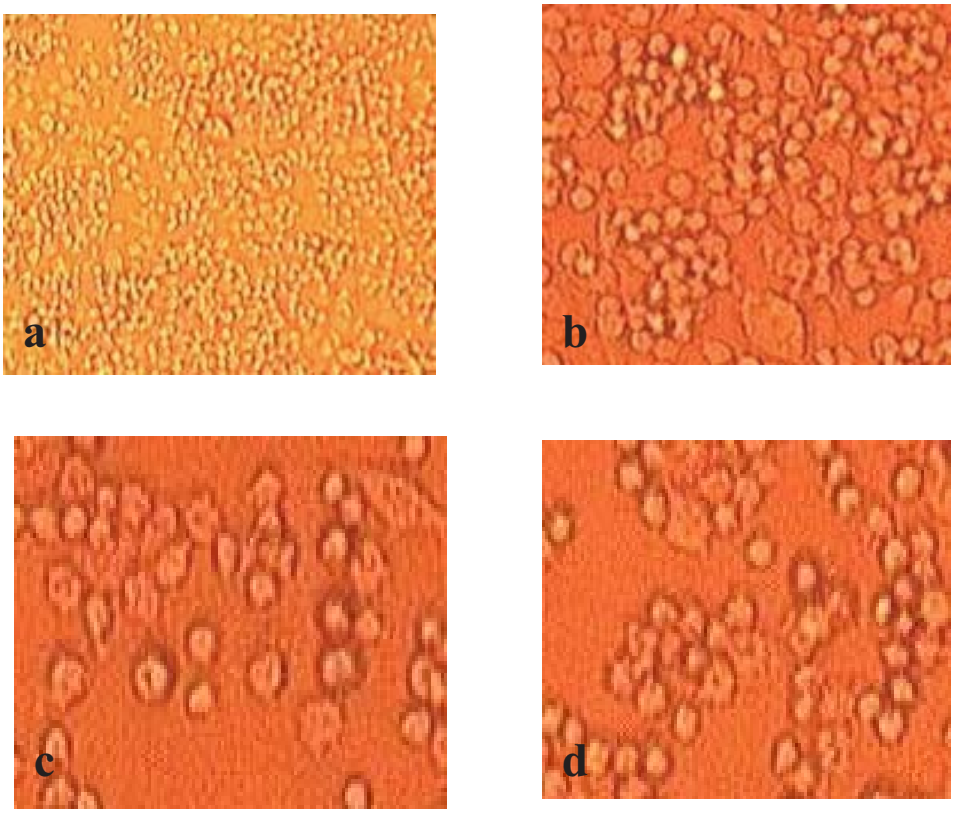
Appendix G Figure A: SEM images of Alveolar Macrophage not exposed to Al-NP 9-28-2006. a) 3300 x magnification, b) 2700 x magnification



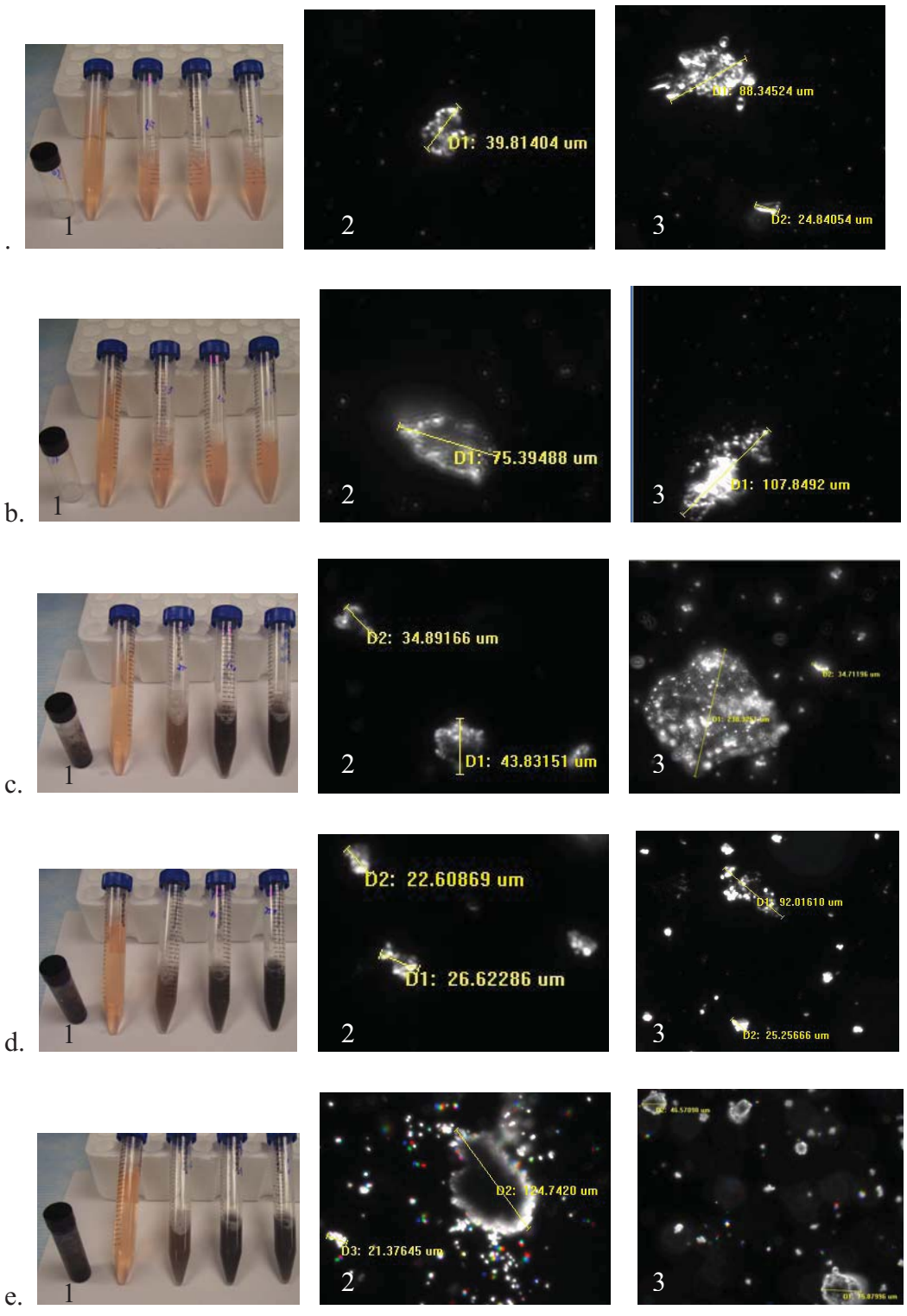
Appendix G Figure B: SEM images of Alveolar Macrophage 24 h post exposure with 50 nm Al-NP at 25 µg/ml on 9-28-2005. a) 4300 x magnification, arrows indicate extensions of plasma membrane. b) 9500 x magnification, ovals indicate possible nanoparticles agglomerates attached to the surface of the plasma membrane.



Appendix G Figure C: SEM images of Alveolar Macrophage 24 h post exposure with 80 nm Al-NP at 25 µg/ml on 9-28-2005. a) 3500 x magnification, arrows indicate possible damage to plasma membrane. b) 6000 x magnification, arrow indicates extension of plasma membrane. c) Area in yellow square in b, arrows indicate possible nanoparticle agglomeration or plasma membrane damage.



Appendix G Figure D: Light Microscopy Images of Rat Alveolar Macrophages (Olympus CK2-001T) a) No aluminum exposure at 20X magnification, cells have a high density and are considered confluent b) No aluminum exposure at 60X magnification, magnification of confluent cells in image a. c) 40 nm Al Oxide exposure at 250 µg/ml for 24 h at 60 X magnification, cell density is noticeably lower than control (images a and b), and less resolution on cell membranes, compared to control, indicating possible membrane damage d) 40 nm Al Oxide exposure at 500 µg/ml for 24 h at 60 X magnification, cell density is noticeably lower than control (images a and b), and less resolution on cell membrane, compared to control, indicating possible membrane damage



Appendix G Figure E: Dispersion and Agglomeration of Al_2O_3-NPs and Al-NPs
a $_{1\text{-}3}$ Al_2O_3 30nm 1) Dispersion from left to right;10mg/ml in de-ionized water (stock solution), Media only, 25µg/ml of 20% FBS media, 100µg/ml of 20% FBS media, 250µg/ml of 20% FBS media. 2) Agglomeration (60X magnification on Olympus IX71

inverted fluorescent microscope and Cyto Viva) 25µg/ml of 20% FBS media. 3) Agglomeration (60X magnification on Olympus IX71 inverted fluorescent microscope and Cyto Viva) 100 µg/ml of 20% FBS media.

b $_{1\text{-}3}$ Al_2O_3 40nm 1) Dispersion from left to right;10mg/ml in de-ionized water (stock solution), Media only, 25µg/ml of 20% FBS media, 100µg/ml of 20% FBS media, 250µg/ml of 20% FBS media. 2) Agglomeration (60X magnification on Olympus IX71 inverted fluorescent microscope and Cyto Viva) 25µg/ml of 20% FBS media. 3) Agglomeration (60X magnification on Olympus IX71 inverted fluorescent microscope and Cyto Viva) 100 µg/ml of 20% FBS media.

c $_{1\text{-}3}$ Al 50nm 1) Dispersion from left to right;10mg/ml in de-ionized water (stock solution), Media only, 25µg/ml of 20% FBS media, 100µg/ml of 20% FBS media, 250µg/ml of 20% FBS media. 2) Agglomeration (60X magnification on Olympus IX71 inverted fluorescent microscope and Cyto Viva) 25µg/ml of 20% FBS media. 3) Agglomeration (60X magnification on Olympus IX71 inverted fluorescent microscope and Cyto Viva) 100 µg/ml of 20% FBS media.

d $_{1\text{-}3}$ Al 80nm 1) Dispersion from left to right;10mg/ml in de-ionized water (stock solution), Media only, 25µg/ml of 20% FBS media, 100µg/ml of 20% FBS media, 250µg/ml of 20% FBS media. 2) Agglomeration (60X magnification on Olympus IX71 inverted fluorescent microscope and Cyto Viva) 25µg/ml of 20% FBS media. 3) Agglomeration (60X magnification on Olympus IX71 inverted fluorescent microscope and Cyto Viva) 100 µg/ml of 20% FBS media.

e $_{1\text{-}3}$ Al 120nm 1) Dispersion from left to right;10mg/ml in de-ionized water (stock solution), Media only, 25µg/ml of 20% FBS media, 100µg/ml of 20% FBS media, 250µg/ml of 20% FBS media. 2) Agglomeration (60X magnification on Olympus IX71 inverted fluorescent microscope and Cyto Viva) 25µg/ml of 20% FBS media. 3) Agglomeration (60X magnification on Olympus IX71 inverted fluorescent microscope and Cyto Viva) 100 µg/ml of 20% FBS media.

Appendix H NIOSH Strategic Plan

Figure A: Strategic Plan for NIOSH Nanotechnology Research: Filling the Knowledge Gaps Excerpt from Appendix A:

TEN CRITICAL OCCUPATIONAL SAFETY AND HEALTH ISSUES ARISING FROM NANOTECHNOLOGY

Projected Timeframe for Addressing Critical Issues

	Calendar Year				
	2005	**2006**	**2007**	**2008**	**2009**
Exposure and Dose	DEP[a] generation and characterization studies (NORA[b]- PRL[c], HELD[d] base) Wildfire ultrafine aerosol and firefighter exposure studies (SNORA[e] -DRDS)[f]	Data and preliminary dosimetry for DEP (HELD base, NORA-PRL) Data and preliminary dosimetry for firefighters (SNORA-DRDS) Dosimetry lung model in rats and humans, begin phase I: structure and calibration w/ existing data (NTRC[g] EID)[h] Initiate TiO_2 workplace exposure assessment (DSHEFS[i] unfunded)	Nanoparticle evaluations in an automotive plant (NTRC-DART[j]) TiO_2 workplace exposure assessment report (DSHEFS unfunded)	Translocation results in laboratory animals after pulmonary and dermal exposure to nanomaterials (NORA-HELD pending) Dosimetry lung model in rats and humans, begin phase II: calibration and validation with translocation data (NTRC-EID pending)	Quantification of systemic nanoparticle concentrations in laboratory animals after pulmonary exposure to nanospheres and nanofibers (NORA-HELD pending)
Toxicity	Preliminary results from toxicity testing in laboratory animal and in vitro systems (NORA-HELD)	Hazard ID information on carbon nanotubes (NORA-HELD)	Preliminary cardiovascular endpoints (NORA-HELD pending) Surface area-mass metric results (NORA-HELD)	Complete dose and time information on carbon nanotubes (NORA-HELD) Nanometal hazard ID (NORA-HELD pending) Dermal information (NORA-HELD pending) Neurological effects (HELD base)	Development of firm cardiovascular endpoints (NORA-HELD pending)

	Calendar Year				
	2005	2006	2007	2008	2009
Epidemiology and Surveillance	Phase I - baseline information gathering (NTRC-DRDS)	Survey of uses and workers (DRDS-DSHEFS unfunded) Phase I - Baseline information gathering (NTRC-DRDS) Correlation of health effects with ultrafine aerosol exposures in auto workers (NTRC-DART-DSHEFS)	Phase I - baseline information gathering Phase II – population studies	Phase I - baseline information gathering Levels of exposure and routes	Further correlation of health effects with ultrafine aerosol exposures in auto workers
Risk Assessment	QRA[k] on TiO_2 from existing studies (EID base)	QRA on other fine and ultrafine materials from existing studies (EID base)	Evaluate QRA methods for nanomaterials, including rodent to human extrapolation and revised dosimetry model (NTRC-EID)	Update QRA for fine and ultrafine particles using new NIOSH data on TiO_2 and carbon black (NTRC-EID pending)	Hazard and preliminary QRA on engineering nanoparticles based on carbon nanotube and ultrafine metal oxide information (NTRC-EID pending)
Measurement Methods	Pilot studies of nanoparticles in the workplace (DRDS) Development of techniques for online surface area measurement (DART)	Measurement studies of nanoparticles in the workplace (DRDS and others)	Established suite of instruments and protocols (NTRC-DRDS) Measurement studies of nanoparticles in the workplace (DRDS and others) Further development of online and offline nanoparticle measurement methods (DRDS-DART)	Viable and practical workplace sampling device for nanoparticles (affordable, portable, effective) (NTRC-DART-DRDS others)	Performance results for nanoparticle measurement instruments and methods (DRDS and others)
Controls	Identification of key control issues	Analyses of filter efficiency (NPPTL-[l] DRDS) Evaluation of control banding options (NTRC-EID- DRDS-DART)	Testing controls in auto plants (DART-DSHEFS) Respirator performance evaluations (NPPTL)	Evaluation of control improvements in auto plants (DART-DSHEFS) Evaluation of clothing and other personal protective equipment (PPE[m])	Summary of control strategies (NTRC-DART)

	Calendar Year				
	2005	2006	2007	2008	2009
Safety	Identification of key safety issues (DSR[n]-NPPTL)		Good safety handling practices (NTRC-DSR-NPPTL)		Summary of nanotechnology safety experience (NTRC-DSR)
Communication and Education	Basic set of FAQs[o] (NTRC, OD[p] base) Web site updates (NTRC-EID, OD, others base) Public presentations (NTRC and many divisions base) NIL[q] pilot (SRL[r]-DRDS)	Expanded set of FAQs (DART, NTRC and many divisions base) Web site updates (NTRC, EID, OD, others base) Public presentations (NTRC and many divisions base) NIL updates (SRL-DRDS)	Web site updates (NTRC, EID, OD, others base) Public presentations (NTRC and many divisions base) NIL updates (SRL-DRDS)	Web site updates (NTRC, EID, OD, others base) Public presentations (NTRC and many divisions base) NIL updates (SRL-DRDS)	Web site updates (NTRC, EID, OD, others base) Public presentations (NTRC and many divisions base) NIL updates (SRL-DRDS)
Recommendations and Guidance	External review of NIOSH TiO_2 CIB[s] (EID base)	Publication of TiO_2 CIB (EID base)	Good working practices guidelines (DART base) Nanomaterials document (NTRC base)	New or updated Nanomaterials document (NTRC base)	New or updated Nanomaterials document (NTRC base)
Applications	NIOSH education series	Roadmap up and running	Prioritized list of short term impact r2p[t]	Further r2p	Further r2p

Abbreviations:

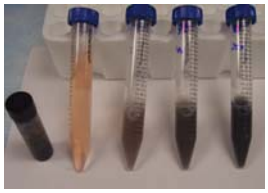
a. diesel exhaust particulate
b. National Occupational Research Agenda
c. Pittsburgh Research Laboratory
d. Health Effect Laboratory Division
e. Small National Occupational Research Agenda on personal protective equipment
f. Division of Respiratory Diseases
g. Nanotechnology Research Center
h. Education and Information Division
i. Division of Surveillance Hazard Evaluation and Field Studies
j. Division of Applied Research and Technology
k. Quantitative Risk Assessment
l. National Personal Protective Technology Laboratory
m. Personal protective equipment
n. Division of Safety Research
o. Frequently asked questions
p. Office of the Director
q. Nano information library
r. Safety Research Laboratory
s. Current intelligence bulletin
t. Research to practice

Appendix I: Proposed Nanoparticle Uptake Methods

Method 1

1

Metal Nanoparticles at known concentrations



Cells Maintained *In Vitro* (alveolar macrophages, karotinocytes etc…)

Dose cells at constant concentration and collect data at 2 h intervals for 24 h0

and

Dose cells at various concentration and collect data at 6 h intervals for 24 h

2

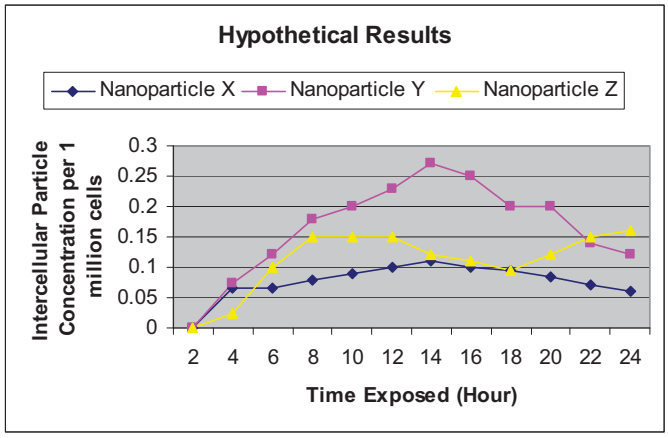
Cell Prep

- Wash Cells PBS, Trypsine, centrifuge
- Cell Count
- Acid digestion of cells

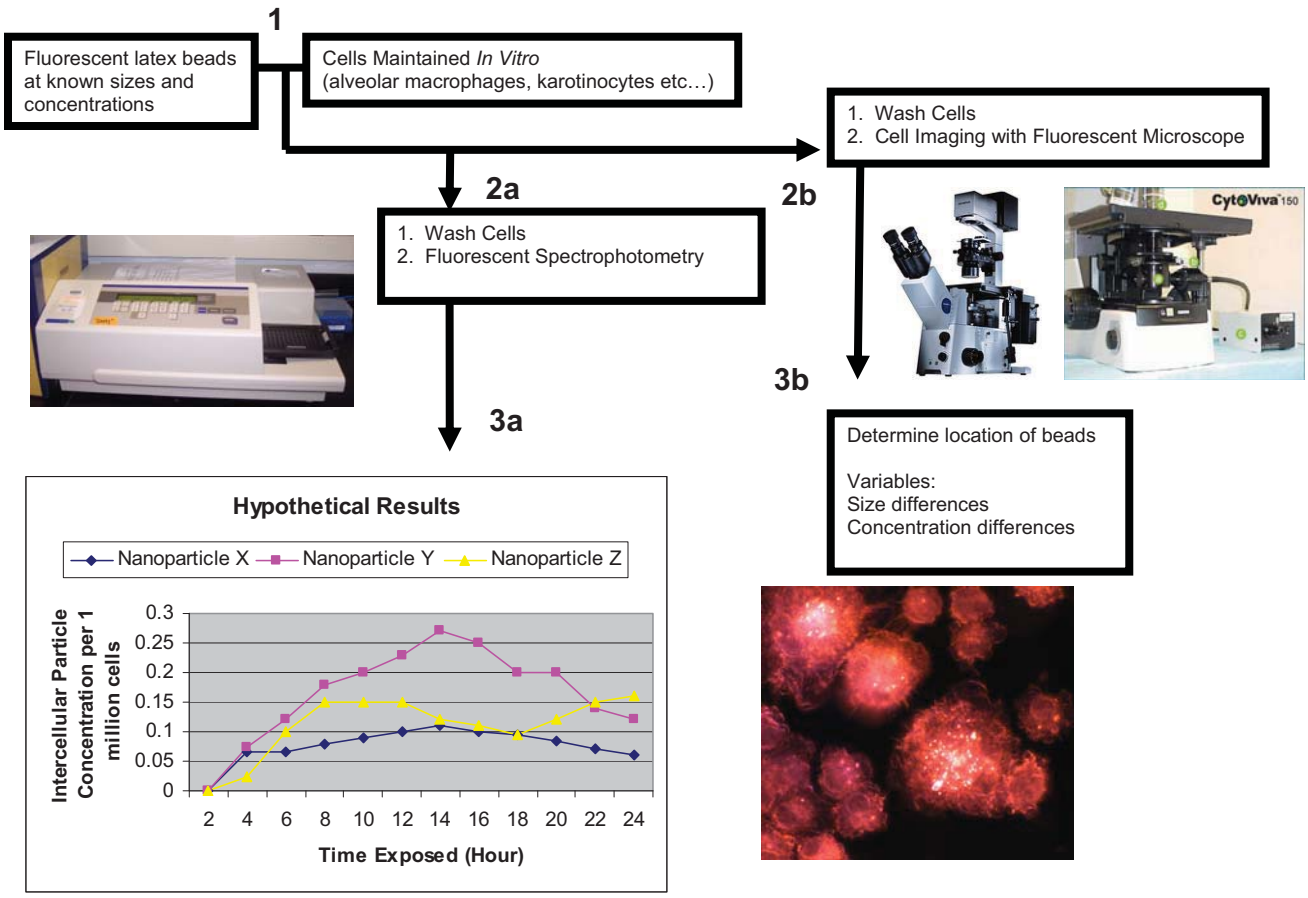
3

Concentration Analysis
1. Atomic Adsorption
2. Inductive Coupled Plasma

4



Method 2



Appendix I: Figure A: Proposed Nanoparticle Uptake Methods

Appendix J :Outline: Proposed Bioenvironmental Role in Nanotechnology

I. Identify Nanotechnology uses across USAF
   A. Current Documents
      1. Defense Nanotechnology Research and Development Programs
      2. Etc
   B. Request for Information from USAF Nanotechnology Working Groups
      1. POC
      2. Material / Particle (Characteristics)
      3. Use
      4. Current number of people exposed
      5. Projected number of people exposed once implemented
      6. Projected implementation
      8. Projected mission impact
      9. Current Controls
      10. Etc.

I. Health Effects
   A. Literature Review
   B. DoD *In Vitro* Data
   C. DoD *In Vivo* Data
   D. DoD Epidemiology Data
   E. Collaborative Data
   F. Etc.

II. Environmental and Work Place Measurement Method Review
   A. Literature Review
   B. Collaboration with Experts
      1. NIOSH
      2. Private Industry
      3. Etc.

III. Risk Assessment
   A. Parameters to consider
      1. Likelihood of use (Answered: I.)
      2. Projected number of people exposed once implemented (Answered: I)
      3. Known Health Effects (Answered: II)
      4. Effectiveness of Control Techniques
      5. Impact on Mission (Answered: I.)
      6. Etc.

   B. Formulate a Prioritized list of Nanoparticles/materials
      1. Recommend toxicology studies from this list
      2. Suggest NIOSH consider modifying its Strategic Plan as needed
      3. AFRL apply for Grants
         a. Environmental and work place measurement methods
         b. Toxicity studies

Appendix K: SEM Protocol

1. Macrophages were seeded on Aclar polymer discs in 24 well plates at 2.5 x 10^5 AM/ml in 20% FBS media for 24 hours

2. The 20% FBS media was suctioned from each well and the cells were washed twice with PBS by gently filling each well with 2mL for 10 minutes then repeating.

3. 1 mL of fixative solution (2.5% glutaraldehyde in 0.1M sodium cacodylate pH 7.4) was added to each well and placed in refrigerator at 4 °C for 1 hour.

4. Fixative was suctioned from each well and washed three times for 15 minutes with 0.1 M Na cacodylate buffer, pH 7.4 (0.2M buffer was diluted with distilled deionized water).

5. Buffer was suctioned and cells were dehydrated in a series of (50, 70, 80, 95, 100, 100, 100%) ethanol. 1mL of ethanol was added for 15 minutes then removed for the next step in the series. The last step in the series was left overnight in a fresh 3 mL of 100% ethanol in a closed dish.

6. Cells were transferred in the 24 well plates in 100% ethanol to the microscopy lab at the University of Dayton.

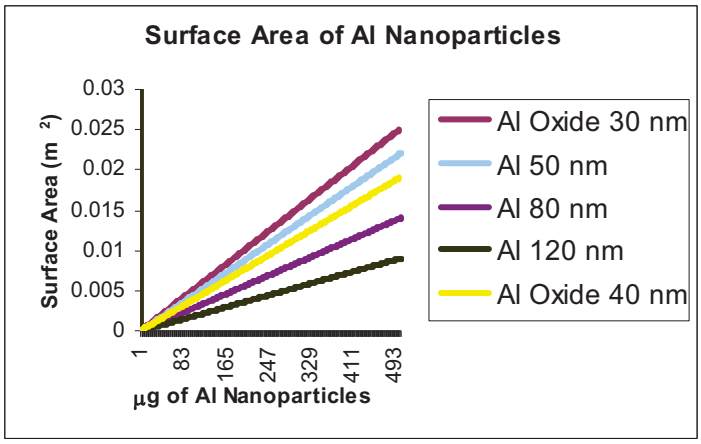
7. The cells were critical point dried and mount to SEM stub.

8. The cells were coated with gold in sputter coater and image in High Resolution SEM. (Gray, 2005) (Schrand, 2005)

Appendix L: Surface Area of Al Nanoparticles

Particle	SSA (m^2/g)
Al Oxide 30 nm	50
Al Oxide 40 nm	38
Al 50 nm	44
Al 80 nm	28
Al 120 nm	18

A



B

Appendix L Figure A: Surface Area of Al Nanoparticles. A) Specific Surface Area provided by Nanotechnologies characterization studies on the materials. B) Calculated Surface Area as mass increased (assuming no agglomeration)

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14. ABSTRACT

The purpose of this research is to characterize the *in vitro* cellular effects of rat lung macrophages to exposure to aluminum oxide nanoparticles (Al_2O_3-NP) (30 and 40nm) compared to aluminum nanoparticles (Al-NP) (50, 80, and 120nm). This study concentrates on cell viability, mitochondrial function, phagocytosis ability, and cytokine response. Results indicate no to minimal toxicological effects on macrophages exposed as high as 500 μg/ml for 24 hours with Al_2O_3-NP. However, there was a significant delayed toxicity that occurred at 96 and 144 h post exposure. Al-NP shows sight to moderate toxicity after 24 h exposure at 100 and 250 μg/ml. The phagocytic ability of these cells was significantly hindered by exposure to each size of the Al-NP at 25 μg/ml for 24 hours, but not by the Al_2O_3-NP. A series of cytokine and nitric oxide assays performed show none of these aluminum nanoparticles are inducing an inflammatory response.

15. SUBJECT TERMS
Nanotechnology, Nanoparticle exposure, Aluminum, Alveolar Macrophage, In Vitro exposure

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